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NOTICE TO CONTRIBUTORS

Beginning with the January 1958 number, ENDOCRINOLOGY will discontinue publication of a summary at the end of each article or note. Instead, an author's abstract will appear at the beginning of each article or note. Effective immediately, manuscripts submitted to ENDOCRINOLOGY must be accompanied by a suitable abstract. The abstract should state as succinctly as possible what was done, what was found and what conclusions were drawn.

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CHANGES IN DOGS DEVOID OF A CELLS[1]

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THE recent and excellent reviews of Cavallero (1), Foa (2) and Korp and Le Compte (3), have indicated that the inability to produce complete and permanent destruction of the A cells of the pancreas has greatly hindered our understanding of their function, particularly as a possible site of origin of glucagon. Recently, several substances have been used in an attempt to destroy the A cells, but there is as yet no general agreement on the exact nature of the A cell lesion thus obtained, nor on the effectiveness of these drugs in various animal species (4, 5, 6, 7, 8). The discovery in our laboratory that the uncinate process of the dog pancreas is normally devoid of A cells, whereas the B and D cells are present (9), and than glucagon is probably absent in this portion of pancreas but abundant in the rest of the organ (10), prompted us to investigate the changes occurring in dogs deprived of their A cells. This was accomplished by means of the selective surgical removal of the A cell-containing portion of the pancreas. It was hoped that this procedure would allow the dogs to produce enough endogenous insulin to prevent the development of diabetes, while at the same time permitting the establishment of a non-complicated A cell deficiency syndrome. In addition, it was hoped that this procedure would yield information on whether the uncinate process could generate A cells once the A cell-containing portion of the pancreas was removed.

MATERIAL AND METHOD

Eighteen healthy mongrel dogs of both sexes, weighing between 25 and 45 pounds, were kept in individual metabolic cages until the completion of the experiment. Animals were weighed about once a week, had free access to water, drank daily about ¼ pint of homogenized milk and ate raw tripe. Pancreatectomized dogs received tripe cleaned of fat to facilitate digestion in the absence of pancreatic juice. Blood samples were obtained from the leg veins about 1½ hours after the morning meal and glucose determined by a micro modification (11) of the Folin-Wu method. Twenty-four hour urine specimens

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were collected in clean vessels to which toluol had been added, and glucose was determined by the quantitative Benedict method (12). Blood and urine sugar were determined approximately twice a week. Glucose tolerance tests were done as follows: glucose (0.3 gm./kg.; in a 20% solution) was injected intravenously in the fasted anaesthetized (Nembutal) dog within 20 to 30 seconds. Blood samples were taken prior to and at 30, 60 and 90 minutes after glucose injection.

For qualitative estimation of the glucagon content of pancreatic tissue, extracts obtained by the method of Best *et al.* (13), were tested according to the method of Staub and Behrens (14). The details of these procedures as performed in this laboratory have been given elsewhere (10). Unless otherwise stated, extracts from 0.5 gm. instead of 1 gm. of pancreas were tested in triplicate whenever sufficient material was available. At the completion of the experiment, dogs were killed by an over-dose of Nembutal. The time at which each dog was killed is indicated in Figure 1. Samples of the following organs were taken at autopsy for histological examination: pituitary, lacrimal gland, thyroid, parathyroid, salivary glands, oesophagus, lung, heart, abdominal aorta, stomach, duodenum, liver, pancreas, spleen, adrenal, gonads, kidney, muscle, sciatic nerve, abdominal skin, abdominal lymph node and sternal bone marrow. Careful search was

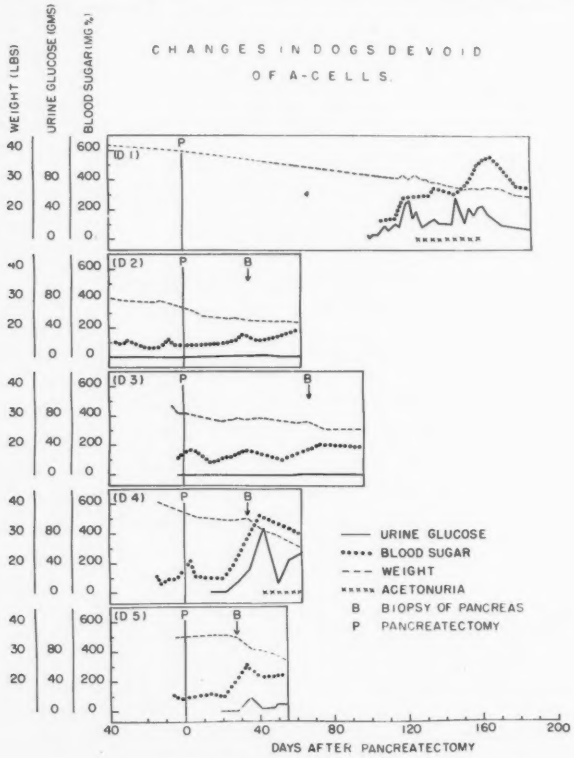


FIG. 1. Changes in weight, blood and urine glucose of partially pancreatectomized dogs devoid of A cells. Biopsy A of the pancreas was taken at the time of partial pancreatectomy (P). Biopsy B was taken at the time of the second surgical intervention (B). Dogs D1, D2, D3, D4 and D5 were killed respectively at 187, 65, 95, 65 and 55 days after partial pancreatectomy.

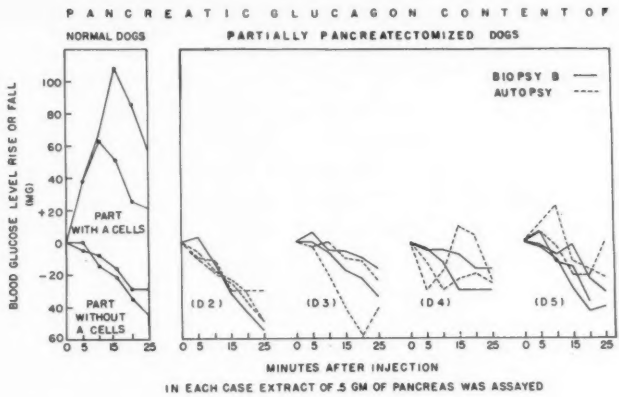


FIG. 2. Shows the lack of hyperglycemic effect of the extract from the normal and the transplanted uncinate process.

made at the site of operation for any residual pancreatic fragments, and all suspect tissue was removed and examined histologically. Tissues were fixed in Zenker formol and formol 10% and processed according to methods previously described; paraffin sections of 2.5 μ were stained with Masson's haemalum-phloxin saffron trichrome method, (15). In addition, pancreas and pituitary were stained with aldehyde fuchsin (16), and with a modification of the chrome-alum-hematoxylin and Masson's trichrome methods (17). Glycogen was stained in the pancreas, lung, liver and kidneys with the Best-Carmine and the Periodic Acid Schiff controlled by diastase digestion.

The animals used in this experiment were divided into two groups. *Group A* (*Control*): Eight untreated dogs served as morphologic controls. In addition extracts for glucagon assay were made from the pancreas of three animals of this group. Extracts from the A cell-free uncinate process and from the A cell-containing parts were pooled. Separately each pooled extract was tested twice, using amounts equivalent to 0.5 gm. (Fig. 2) and 1 gm. of pancreas (Table 1). *Group B* (*Partial pancreatectomy*): All surgical procedures were performed under Nembutal anaesthesia. In 10 dogs, body and tail of the pancreas were removed and the uncinate process transplanted subcutaneously. A pancreatic fistula was created through the skin in order to prevent atrophy of the islet tissue which otherwise would occur in the transplant (19). To make sure that the transplanted portion of the pancreas was devoid of A cells, a complete transverse section of the uncinate process proximal to the body of the pancreas was taken (biopsy A) for histological examination at the time of transplantation. Previous work performed in this laboratory has shown that this procedure will tell invariably whether A cells are present in the region distal to the biopsy (9, 10). In 2 dogs, the two-stage pancreatic transplant procedure of Ivy *et al.* (20) was followed in full. In one of these animals (D1) the pancreatic transplant became completely dependent for its blood supply on the vascular connections established with the subcutaneous vessels. In the other 8 animals, the operative procedure was modified allowing the original vascular pedicle of the transplanted uncinate process to remain intact. Five of the operated dogs (1 female, D1, and 4 males, D2, D3, D4 and D5) survived the experimental period; the others died of various post-operative complications, (pneumonia, eventration, etc.).

To study the early histological changes in the transplanted uncinate process in dogs D5, D4, D2, D3 and D1, a biopsy B was obtained from the transplant at 28, 34, 35, 67 and 187 days respectively after removal of the remaining portion of the pancreas. This biopsy required cutting of the abdominal vascular supply to the transplant in

TABLE 1. BLOOD SUGAR RESPONSE OF FASTING ANAESTHETIZED NORMAL CATS TO INTRAVENOUS INJECTION OF DOG PANCREATIC TISSUE

Material injected	Dose,* gm.	Blood sugar, mg. %					
		Before inj.	Min. after admin. of extract				
			5′	10′	15′	20′	25′
Extract from the transplanted uncinate process of dog D1	1	111	149	123	120	111	123
	1	100	129	120	105	100	89
	1	105	141	130	105	105	105
	1	108	143	130	120	105	105
	1	104	108	116	102	100	80
Control	1	125	156	170	172	148	103
A. Extract of A cell containing portion of pancreas (pool of three control dogs)							
B. Extract of pancreatic tissue devoid of A cells (uncinate process) (pool of three control dogs)	1	138	119	115	106	91	85

* Calculated in term of wt. of fresh pancreas.

order to preserve the pancreatic fistula. Thus the blood supply of the remaining part of the transplant became dependent on the newly established vascular connection with the skin vessels, becoming comparable, in this respect, to the pancreatic transplant of dog D1. The amount of tissue resected for biopsy B in each dog is given in Table 2. A complete transverse section was used for microscopic studies, while the remainder was used for glucagon extraction in all dogs, but D1. At the end of the experiment, immediately after killing the animals, identical studies were made on the remaining part of the pancreatic transplant.

In dogs D2, D3 and D4, one glucose tolerance test was done before partial pancreatectomy and another one week before the second pancreatic biopsy. In dog D5, the second glucose tolerance test was not done.

RESULTS

Group A (Controls): In agreement with our previous findings (9, 10), the uncinate process contained B, D and X cells, but not A cells and its extracts were devoid of hyperglycemic activity (Fig. 2 and Table 1). In one dog, at the junction between the body and the uncinate process, there was gross evidence of incomplete embryological fusion and microscopic examination revealed a sharp demarcation line (Figs. 3, 4 and 5). Unex-

TABLE 2. MORPHOLOGICAL CHANGES IN THE TRANSPLANTED UNCINATE PROCESS OF DOGS DEPRIVED OF A CELLS BY SELECTIVE PARTIAL PANCREATECTOMY

Dog	B-cell degran.			Glycogen infiltration						Pancreatic fibrosis			Pancreas used for gluc. ext		Total trans.
				B-cells			Duct cells						Gm.		Gm.*
	Biop.		Autop.	Biop.		Autop.	Biop.		Autop.	Biop.		Autop.	Biop.	Autop.	
	A	B		A	B		A	B		A	B		B		
D1	0	—	4	0	—	4	2	—	4	0	—	1	—	5.8	5.8
D2	0	1	3	0	0	1	1	2	3	0	0	1	1.7	5	6.7
D3	0	1	2	0	0	0	1	1	2	0	±	1	2	5	7
D4	0	3	4	0	1	4	2	2	4	0	3	0	1.5	2	3.5
D5	0	1	3	0	0	1	2	2	3	0	1	1	4	2	6

* These values do not include the weights of the tissues used for histological examinations for the 2nd biopsy and the autopsy. The combined weights for both these two sections was approximately 1 gm.

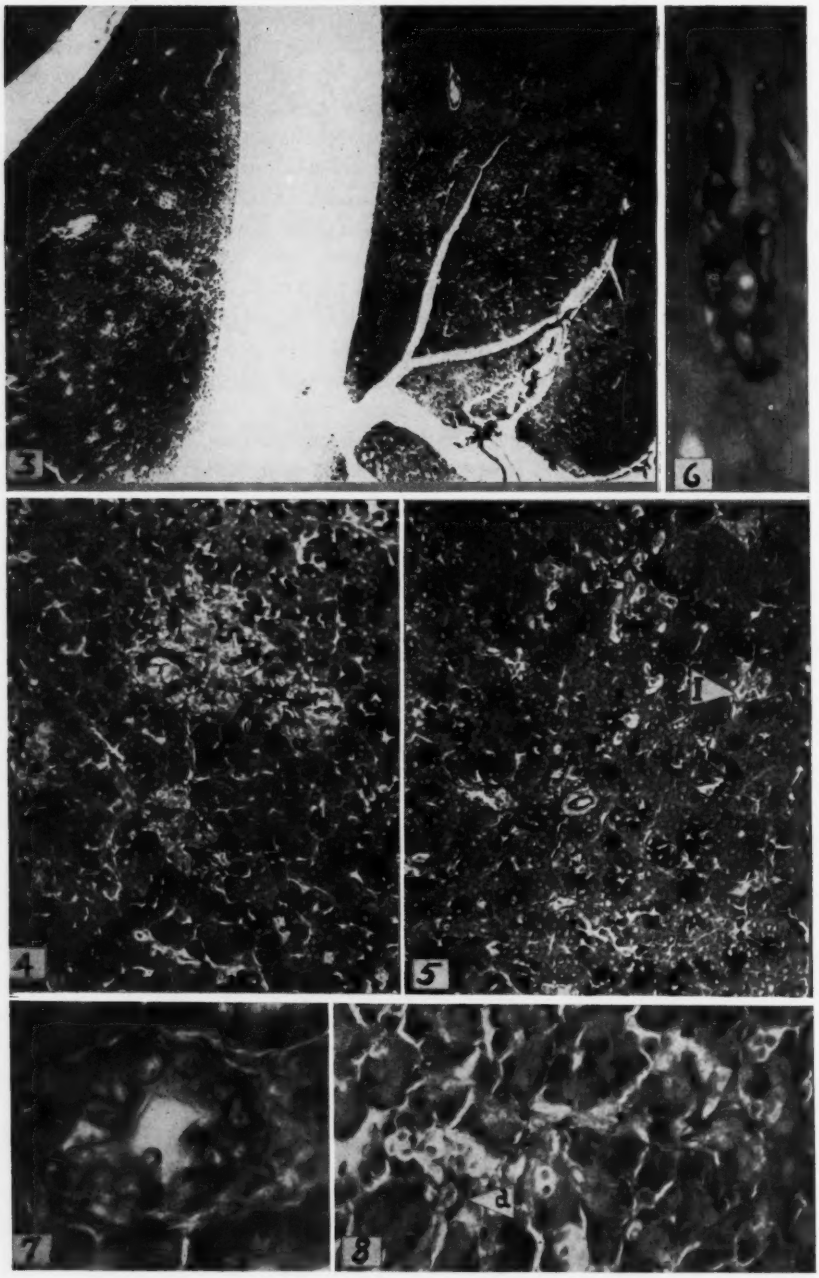
pected but interesting was the finding of glycogen in the small and medium-sized pancreatic ducts of all control dogs (Figs. 6, 7 and 8). It was also noticed that many of the tubular cells of the kidney contained large amounts of histochemically demonstrable glycogen.

Group B (Partial pancreatectomy): Little changes were noted in blood and urine glucose of dogs D2, D3, and D5 after partial pancreatectomy, while in D4 a mild diabetes appeared (Fig. 1). On the other hand the post-operative glucose tolerance test was invariably diabetic in type (Fig. 9), except for dog D5, in which the test was not done. Variable glycosuria and hyperglycemia appeared in all dogs after biopsy B, and were most severe in dogs D4 and D5 which had the smallest transplant. Dog D4, which showed the greatest weight loss and highest hyperglycemia and glycosuria, developed a permanent ketonuria (trace) about one week after biopsy B (Table 2 and Fig. 1).

The subcutaneous pancreatic fistula was functioning in all dogs until the time of death. However, it seems that mild pancreatic duct obstruction was present in most instances, as suggested by minimal to moderate fibrosis and diffuse duct hyperplasia found in biopsy B and at autopsy (Fig. 10). The intensity of these changes is given for each case in Table 2 under the heading of "pancreatic fibrosis." Only in dog D4, a microscopic fragment of pancreas was found attached to the duodenal wall, probably remaining from the time of pancreatectomy. This showed well preserved acinar tissue, ducts cells markedly infiltrated with glycogen, and two small accumulations of 10–15 cells which were probably islet cells. However, no A cells were seen in the numerous serial sections obtained from this area.

In all dogs, biopsy A confirmed the fact that the transplant was devoid of A cells. Variable amounts of glycogen were found in biopsy A in all dogs (Table 2 and Figs. 6, 7 and 8). In no case were A cells found in biopsy B, or in the remnant of the pancreatic transplant obtained at autopsy, nor was the structure of the D and X cells altered in these two sections of the pancreas. Some degranulation of the B cell and variable amounts of glycogen infiltration of both duct and B cells were observed in the operated dogs (Figs. 10, 11 and 12). The intensity of these changes in both biopsy B and in the pancreatic tissue removed at autopsy is given for each animal in Table 2. The degree of glycogen infiltration of the B cells could be roughly correlated with the severity of hyperglycemia and glycosuria present in these operated dogs. Of interest was the presence in the pancreas of these dogs of neuro-insular complexes containing X cells, and showing glycogen infiltration in the B cells (Fig. 13).

It is difficult to say when diabetes began to appear in dog D1 since no biochemical studies were made before the 100th day after partial pancreatectomy. Between the 100th and 120th day after operation very small hyperglycemia and glycosuria were noted and permanent diabetes became well established after the 120th day. On the 126th day this dog developed acute ketosis which was successfully treated with a single dose of 20 units



FIGURES 3–8

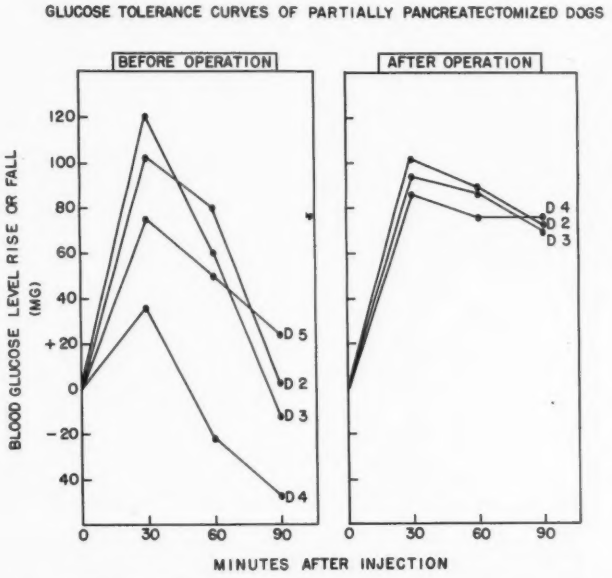


FIG. 9. Shows that all partially pancreatectomized dogs had a diabetic type of glucose tolerance.

of protamine zinc insulin (Conaught Laboratories). Traces of acetone, however, continued for a few weeks, then cleared spontaneously (Fig. 1). Until the time of death, the subcutaneous pancreatic fistula remained functional. At the time of autopsy, the pancreatic tissue of dog D1 showed severe degranulation of B cells and glycogen infiltration of B cells and ductular epithelium. No changes were found in the X or in the D cells. Because of

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EXPLANATION OF FIGURES 3–8

FIG. 3. General view at the site of junction of the ventral and dorsal pancreas in one dog which presented the incomplete fusion of these two portions of the pancreas. On the left side is the portion of pancreas with large islets containing A cells, whereas on the right side, no islet can be distinguished with this magnification. Aldehyde fuchsin. ×20.

FIG. 4. High power from Figure 3 showing the zone containing A cells. Note the large size of islets when compared with those of Figure 5. ×100.

FIG. 5. High power from Figure 3 showing the zone devoid of A cells. ×100.

FIG. 6. Uncinate process of dog D1 at the time of the partial pancreatectomy (biopsy A). Glycogen in duct cells. PAS. ×580.

FIG. 7. Body of the pancreas of dog D1 at the time of the partial pancreatectomy (biopsy A). Glycogen in duct cells. PAS. ×580.

FIG. 8. General view of the uncinate process of dog D1 at the time of partial pancreatectomy (biopsy A). Numerous cells of the intercalated ducts (d) heavily stained with the PAS stain. ×384.

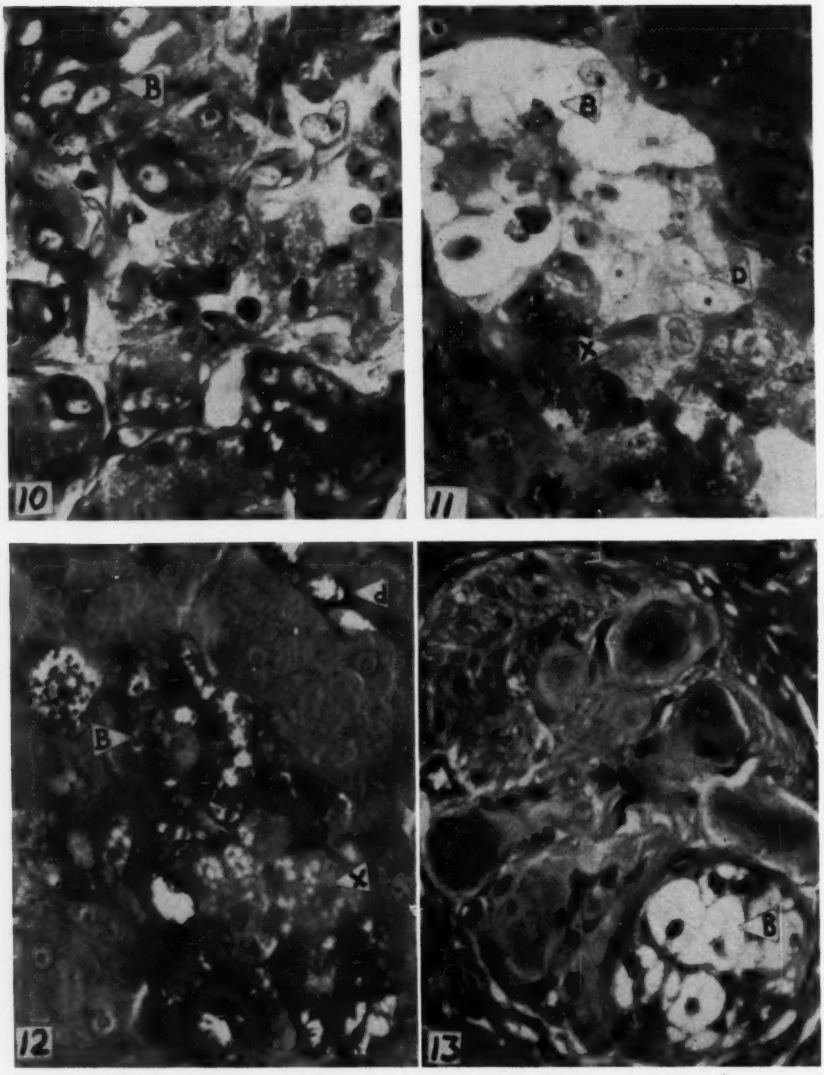

FIG. 10. Biopsy B of dog D4 showing disruption of the normal structure of acini and the presence of a few islet cells with marked glycogen infiltration. PAS. ×580.

FIG. 11. Uncinate process of dog D1 obtained at autopsy, showing marked hydropic degeneration of the B cells, normal appearing non-granular D cells and coarsely granulated X cells. Masson's trichrome. ×580.

FIG. 12. Uncinate process of dog D4 obtained at autopsy, showing marked glycogen infiltration of B cells and intact X cells. The small vacuolization of these cells is of normal occurrence in them. There is also marked glycogen infiltration of a small duct (d). PAS. ×580.

FIG. 13. Neuro-insular complex containing B cells with marked hydropic degeneration. ×580.

the results of the glucagon assay in this pancreas, 200 serial sections were examined and only two cells were observed which stained like A cells.

Bio-assay of pancreatic extract: As seen in Figure 2, no significant hyperglycemic effect was induced with extracts obtained from biopsy B, or from the remaining portion of the transplanted uncinate process removed at autopsy, from dogs D2, D3, D4 and D5 (Fig. 2). Pancreatic extracts from these dogs elicited similar hypoglycemic effects in the cat, regardless of whether the B cells were hydropic, or slightly or severely degranulated (Fig. 2). The pancreatic extracts from the transplanted pancreas of dog D1 elicited a moderate initial hyperglycemia (Table 1) which was followed by almost no hypoglycemia, despite the fact that the pancreas obtained at the time of autopsy from dog D4 was histologically indistinguishable from the pancreas of dog D1.

In general, the extrapancreatic changes observed in all treated dogs were similar to those found in other types of experimental diabetes: glycogen infiltration (hydropic change) was considerably greater in the kidney tubules of dogs D1, D4 and D5 than in the controls; liver glycogen was normal in Dogs D2 and D3, decreased in the other animals. The livers of dogs D1 and D4, which had the most severe diabetes, showed moderate and minimal centrolobular fatty change respectively. There was marked reduction of spermatogenesis in dogs D3, D4 and D5. In the seminiferous tubes of D4 and D5, one could see a few giant cells, similar to those described in animals with some nutritional deficiencies (21).

DISCUSSION

Despite the absence of A cells in the experimental animal no A cell developed in the transplanted uncinate process during the period of 28 to 187 days. Since during this time, neither the X nor the D cell changed in structure or in their relative number, one may conclude that the A, B, D and X cells are histogenetically and functionally independent cells, that they are not capable of differentiating from one to another and finally, that the uncinate process has no precursors for the A cells. This is in agreement with previous work on the histogenesis of islet cell in the rabbit (6, 7). It is possible, however, that the procedure used did not provide the proper stimulus for the formation of A cells in the uncinate process or that perhaps residual fragments of pancreas containing A cells became hyperactive and compensated for the A cells removed in these operated animals. The latter explanation does not seem tenable since careful exploration at autopsy failed to reveal any fragment save for a minute amount in one case.

The changes observed in the B cell and duct cells of dogs deprived of their A cells did not differ from those described in animals subjected to conventional subtotal pancreatectomy; degranulation of B cells appeared in our operated dogs at about the same time, and showed a similar relation to the height and duration of the hyperglycemia, as was described in

the work of Allen (22), suggesting that the changes in the B cells did not require the presence of A cells nor of glucagon in order to develop.

The presence in normal dogs of histologically demonstrable glycogen in the epithelium of small and medium-sized pancreatic ducts is of considerable interest. Perhaps the greater degree of glycogen infiltration seen in the duct cells of diabetic animals might represent some sort of exaggeration of a normal function (i.e., post-prandial hyperglycemia?), rather than a degenerative phenomenon. Glycogen was never seen in the B cells of the control dogs.

The type of pancreatectomy carried out in this group of animals is such that there is a complete elimination of A cells, and a diminution in B cell content. The conventional pancreatectomy reported by others results in a porportional diminution of both A and B cell elements. Since the type and severity of diabetes in the two experimental procedures proved to be similar, one may reasonably cast doubt on the diabetogenic influence of the A cells in the dog. In view of the supposed origin of glucagon from the A cells (10, 23, 24) and the known diabetogenic activity of this agent in rats (25) further studies on the metabolic effects of glucagon in the dog are required.

The lack of alterations in the pituitaries of dogs deprived of A cells is difficult to reconcile with several reports in which a relationship between the anterior pituitary and these cells had been stated to occur (1, 2, 3), unless adaptive mechanisms have occurred to mask this relationship at the morphological level. The presence of peculiar giant cells in the testes of two dogs and of marked diminution of the spermatogenesis in all operated male animals was probably due to nutritional deficiencies related perhaps to the absence of pancreatic juice, rather than to a lack of A cells, since similar lesions have been found in other nutritional deficiencies (21).

SUMMARY

A procedure for the removal of the A cells of the pancreas by means of a selective partial pancreatectomy has been described. This permits metabolic studies in dogs devoid of A cells without the accompaniment of frank diabetes.

Despite the removal of the A cell-containing portion of the pancreas, no A cells appeared in the transplanted uncinate process which is normally devoid of A cells, even though the operated dogs were observed for a period as long as 187 days. No changes were found in the D and X cells of the transplanted uncinate process. No hyperglycemic materials were found in the extracts of transplanted uncinate process of all dogs except one, which had a moderate hyperglycemic effect.

Some time after partial pancreatectomy, B cells underwent progressive degranulation and glycogen infiltration. These changes were greatest in animals with the smallest uncinate transplant and were associated with a

diabetic condition as revealed by a decreased tolerance to glucose and eventually by the development of hyperglycemia and glycosuria. With the method used here, the diabetic syndrome in dogs devoid of A cells was essentially similar to that described in dogs partially pancreatectomized by the conventional method where many A cells remained. Morphological alterations outside the pancreas were in general no different from what one finds in other types of experimental diabetes.

In conclusion, there is strong evidence to support the idea that glucagon is produced by A cells and that the A, B, D and X cells are independent cells which do not convert into one another, even when all pancreatic A cells have been removed.

Although no clear-cut A cell deficiency syndrome has been produced, the method described may prove useful for studying the physiological significance of the A cell.

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INHIBITION OF PITUITARY HORMONE ACTIVITY WITH DERIVATIVES OF PITUITARY PREPARATIONS[1]

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A PREVIOUS publication (1) reported that the administration of a sulfur labeled thyrotrophic hormone preparation failed to cause an increase in thyroid weight, although a significant localization of radioactivity occurred in the thyroid gland. Thus, the biological activity of a pituitary trophic hormone (TSH) apparently could be dissociated into at least two properties: one localizing, the other stimulatory. Subsequently, it was found[3] that there was a decreased localization of the administered radioactive sulfur in the thyroid when an S^{35} labeled TSH preparation was administered with increasing amounts of unmodified TSH. If the S^{35} found in the thyroids represented thyrotrophic hormone, this latter observation might suggest that there is a limited number of binding sites available at any one time in the thyroid for localization of TSH.

On the basis of the above observations, a theory of inhibitory action was formulated. It was assumed that the thyroid contains a limited number of binding sites available for TSH localization. In addition, it was postulated that a TSH preparation unable to promote growth of the thyroid but capable of localizing at these sites might block the localization of endogenously secreted or exogenously administered TSH. This then would suggest that pituitary derivatives might be useful as inhibitors of hormone activity. Preliminary experiments with pituitary preparations esterified with concentrated sulfuric acid suggested some inhibition of thyrotrophic stimulatory activity. Such preparations, however, had undesirable physical properties and were toxic when tested in experimental animals. For this reason, a systematic study was made of the derivatives of pituitary protein preparations containing thyrotrophic and gonadotrophic activity. These derivatives were prepared on the basis of known functional groups of proteins. Thirteen different types of derivatives were prepared. In many cases, with each type of derivative, several preparations were obtained after variation of the reaction conditions and the stoichiometry.

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[2] American Cancer Society Scholar.

[3] Sonenberg, M. and Money, W. L. To be published.

Of the many derivatives tested, only three showed inhibitory properties for thyrotrophin. The present publication is an amplification of a previous report (2) of studies with acetylated pituitary preparations which originally contained thyrotrophic and gonadotrophic hormonal activity.

METHODS

A. *Chemical*

1. *Preparative*

2 gm. of a pituitary preparation[4] containing thyrotrophic and gonadotrophic activity were suspended in acetic anhydride. Various concentrations of pituitary preparation in acetic anhydride ranging from 0.25% to 4% were used and the reactions were carried out both in the cold (0–2° C) and at room temperature (21–27° C). Upon addition of the hormone preparation, the mixtures were stirred constantly, in different experiments for from one to 300 minutes. Following this reaction period, a volume of distilled water equal to that of the acetic anhydride was added with constant stirring for 5 minutes and the dissolved acetylated preparation was dialysed against cold (4° C) running tap water for 6 days and then freeze-dried. (Approximately 1.9 gm. of salt-free material were obtained).

An acetylated bovine serum albumin preparation was similarly obtained by suspending bovine serum albumin in acetic anhydride.

2. *Analytical*

Volatile acid groups were determined by a modification of the Elek and Harte method (3).[5]

Amino nitrogen of the unmodified and acetylated protein preparations was determined by the method of Van Slyke and Birchard (4).

B. *Biological*

For the evaluation of thyrotrophic and gonadotrophic stimulatory and inhibitory activity, one-day old cockerels[6] were used. In the various experiments, each animal received doses ranging between one and 125 mg. of an acetylated pituitary preparation over a 6-day period. During the same period of time, other groups of animals were given in addition to the acetylated preparation an unmodified thyrotrophic hormone preparation. Other groups of animals received only the thyrotrophic hormone preparation, or served as uninjected controls. On the seventh day, each animal was injected subcutane-

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[4] We should like to thank Parke, Davis and Company and the Armour Company for making some of these preparations available to us.

Biological assay of the preparations employed in this study indicated they contained approximately the following thyrotrophic hormone content expressed as U.S.P. units per milligram.

PD 494 E 15—0.5
PD 50 P 4 —2.5
Armour 264 —0.5
PD 099861 —2.5
PD X6585 —2.5

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[6] We would like to express our appreciation to Mr. Monroe Babcock of the Babcock Poultry Farm, Inc., Ithaca, New York for the generous supply of white Leghorn cockerels used in these studies.

ously with doses ranging between one and 0.1 microcurie of radioactive iodine (I^{131}). On the eighth day, all were sacrificed with ether anesthesia, the thyroids and gonads were removed, dissected free of adjacent tissue and weighed. The thyroids were placed in 5 ml. screw-cap vials containing one ml. of 0.1 N NaOH and the radioactivity in these vials was determined in a well-type scintillation counter against a standard.

The results of the radioactivity determinations have been expressed both as the per cent of the injected I^{131} which localized per thyroid, and per mg. of thyroid tissue. The thyroid and testes weight has been represented as both the absolute weight and the ratio of organ weight per 100 gm. of body weight.

The data have been evaluated statistically according to the method of Fisher (5). In all experiments the group receiving the pituitary preparation (AP Prep.) has been compared to the uninjected controls (no treatment). The group receiving the experimental substance and pituitary preparation has been compared with AP Prep. alone. The group injected only with the experimental material was contrasted with the uninjected controls.

RESULTS

Although many experiments were done, for the sake of brevity representative data are included.

The dose of an unmodified pituitary preparation necessary to induce a doubling in thyroid weight and a tripling in testes weight was found to be between one and 2.5 mg. for purified material or 20 mg. of a crude pituitary extract.

The results obtained with one preparation acetylated with acetic anhydride at 1° C for 4 hours are summarized in Table 1. When this material was tested in another experiment in amounts as high as 125 mg., no thyroid or testes growth was observed. Thyroids of animals receiving the acetylated pituitary preparation alone were slightly smaller (3.1 mg.) than those (4.4 mg.) from an untreated group of chicks. When this preparation was administered in doses approximately 4.6 times the dose of a simultaneously administered unmodified pituitary preparation, the resultant chick thyroid weight (5.3 mg.) was significantly reduced when compared to a group receiving only the unmodified pituitary preparation (11.1 mg.). There was, however, essentially the same increase in chick testes weight when the unmodified preparation was administered, with or without, the acetylated

TABLE 1. THE EFFECT OF AN ACETYLATED PITUITARY PREPARATION ON THE THYROID AND TESTES WEIGHT AND THE THYROIDAL UPTAKE OF RADIOACTIVE IODINE

Type of treatment total dose (mg.)†	No. of chicks	Body wt. (gm.)	Thyroid wt. (mg.)	Thyroid wt./ (100 gm. body wt.)	I^{131} uptake		Testes wt. (mg.)	Testes wt./ (100 gm. body wt.)
					%/dose	%/mg.		
AP. Prep. (20)	10	68	11.1* ±1.1	16.3*±1.2	14.3*±1.2	1.4* ±0.1	30.5*±1.6	44.8*±2.3
AP. Prep. (20) & TI 34 (91)	7	58	5.3* ±0.6	9.1*±0.2	9.6*±1.3	1.7* ±1.9	26.7 ±5.4	46.0 ±7.4
TI 34 (91)	9	63	3.1**±0.6	4.9*±0.2	1.3*±0.2	0.5**±0.1	13.7 ±2.3	21.7 ±2.0
No Treatment	9	66	4.4 ±0.2	6.7 ±0.4	2.4 ±0.8	0.6 ±0.1	13.3 ±1.3	20.1 ±1.9

AP. Prep.—Pituitary preparation (Parke, Davis 494 E15) with thyrotrophic and gonadotrophic activity.
TI 34 —Pituitary preparation (Parke, Davis 494 E15) acetylated at 1° C for 4 hours. The number is merely a code.
* —Indicates a p value of 0.01 or less.
** —Indicates a p value between 0.05 and 0.01.
†( ) —Indicates total dose in mg. or ml.

pituitary preparation. Acetylation of this pituitary preparation not only destroyed its ability to increase thyroid weight but also the ability to increase the thyroidal uptake of radioactive iodine. Moreover, the thyroid accumulation of I^{131} in animals treated with preparations of unmodified TSH and 91 mg. of acetylated TSH was less (9.6%) than found in chicks treated with unmodified TSH alone (14.3%).

The administration of acetylated bovine serum albumin prepared in a manner identical with the acetylated pituitary preparation, failed to modify the response of the chick testes and thyroid, with respect to weight or I^{131} accumulation, to the simultaneously injected pituitary preparation (Table 2). Moreover, such a preparation did not change the weights of the testes and thyroid or I^{131} uptake of chicks receiving no exogenous TSH. Although a 1–100 dilution of glacial acetic acid did not modify the response of the thyroid or I^{131}, the testes weight of such treated animals was sig-

TABLE 2. THE EFFECT OF AN ACETYLATED BOVINE SERUM ALBUMIN PREPARATION AND AN ACETIC ACID SOLUTION ON THE THYROID AND TESTES WEIGHT AND THE THYROIDAL UPTAKE OF RADIOACTIVE IODINE

Type of treatment totsl dose (mg. or ml.)†	No. of chicks	Body wt. (gm.)	Thyroid wt. (mg.)	Thyroid wt./ (100 gm. body wt.)	I^{131} uptake		Testes wt. (mg.)	Testes wt./ (100 gm. body wt.)
					%/dose	%/mg.		
AP. Prep. (1)	10	64	5.4*±0.7	8.4*±0.9	25.9*±3.1	4.9*±0.4	35.3*±3.8	55.2*±5.0
AP. Prep. (1) & BSA 18 (22)	9	66	5.5 ±0.5	8.3 ±0.8	25.8 ±3.6	4.6 ±0.4	35.8 ±3.6	54.2 ±5.0
BSA 18 (22)	10	69	3.2 ±0.2	5.0 ±0.3	5.2 ±0.8	1.7 ±0.2	18.6 ±1.7	27.0 ±2.0
AP. Prep. (1) & HAc (6)	9	65	7.0 ±0.9	10.8 ±1.2	32.4 ±6.7	4.4 ±0.5	42.2 ±4.6	64.9*±5.4
HAc (6)	10	65	3.3 ±0.2	5.1 ±0.7	3.4 ±0.7	1.1 ±0.2	12.4*±1.3	19.1*±0.2
No Treatment	10	70	3.1 ±0.3	4.4 ±0.3	4.2 ±0.8	1.3 ±0.2	18.1 ±2.2	25.9 ±2.1

AP. Prep.—Pituitary preparation (Parke, Davis 50 P4) with thyrotrophic and gonadotrophic activity.
BSA 18 —Bovine Serum Albumin Preparation (Armour 212-106) acetylated at 1° C for 4 hours.
HAc —Acetic Acid Solution (1 to 100 dilution of Glacial Acetic Acid).
* —Indicates a p value of 0.01 or less.
†( ) —Indicates total dose in mg. or ml.

nificantly less than control values. A pituitary preparation (Armour 264) when reacted at 24° C and another pituitary preparation (Parke, Davis 50 P4) when reacted at 1° C required between 30 and 300 minutes of reaction to obtain a product which was able to inhibit the thyroid response to an exogenously administered TSH preparation (Table 3). In most cases, the ability of the pituitary preparations to cause an increase in thyroid weight was essentially lost upon reacting with acetic anhydride in either the cold, or at room temperature between one and 300 minutes. Variation, however, in the time of exposure of different pituitary preparations to acetic anhydride did not impart inhibitory properties against exogenously administered gonadotrophins whether the results were expressed as absolute testes weight or as testes weight per 100 gm. of body weight (Table 3). Acetylated pituitary preparations, obtained by reacting with acetic anhydride at 24° C, when administered without the concomitant injection of unmodified pituitary preparations did result in smaller testes weight. Acetylation of a pituitary preparation (Armour 264) gave a product where there was complete loss in gonadotrophic activity with all reaction times

TABLE 3. THE RELATIONSHIP OF REACTION TIME OF PITUITARY PREPARATIONS AND ACETIC ANHYDRIDE TO THE STIMULATORY AND INHIBITORY ACTIVITY OF THESE PREPARATIONS

Reaction time (min.)	No. of chicks	Type of treatment total dose (mg.)†	Body wt. (gm.)	Thyroid wt. (mg.)	Thyroid wt./ (100 gm. body wt.)	I^{131} uptake		Testes wt. (mg.)	Testes wt./ (100 gm. body wt.)
						%/dose	%/mg.		
1	10	AP. Prep. (1) & TI 102(35)	72	7.5 ±0.7	10.4 ±0.9	13.3 ±2.1	1.7 ±0.2	34.0 ±2.6	47.2 ±4.4
1	10	TI 102(35)	69	4.0 ±0.2	5.8 ±0.3	1.9 ±0.3	0.5 ±0.1	15.7* ±1.6	22.8* ±1.2
5	10	AP. Prep. (1) & TI 103(35)	64	7.3 ±0.9	11.4 ±1.3	12.0 ±1.3	1.7 ±0.1	28.2 ±2.7	44.1 ±3.5
5	10	TI 103(35)	67	4.5 ±0.3	6.7 ±0.4	1.9 ±0.4	0.4 ±0.1	17.0* ±1.6	25.4* ±2.0
30	9	AP. Prep. (1) & TI 104(35)	64	5.0**±0.7	7.8**±0.8	6.2**±1.7	1.1 ±0.2	30.9 ±3.1	48.3 ±3.1
30	10	TI 104(35)	70	3.7 ±0.3	5.3**±0.3	1.7 ±0.4	0.4 ±0.1	19.0 ±1.8	27.1**±2.1
300	9	AP. Prep. (1) & TI 105(35)	63	4.7* ±0.4	7.5* ±0.6	8.9 ±1.3	1.8 ±0.3	27.6**±2.1	43.8 ±4.1
300	10	TI 105(35)	58	3.4**±0.4	5.9 ±0.5	1.2 ±0.3	0.3 ±0.1	14.5**±2.0	25.0**±2.8
1	8	AP. Prep. (1) & TI 106(35)	70	6.3 ±0.7	9.0 ±1.1	9.1 ±1.1	1.5 ±0.1	41.3 ±5.8	58.8 ±6.2
1	10	TI 106(35)	67	3.7 ±0.3	5.5 ±0.5	1.8 ±0.4	0.5 ±0.1	30.7* ±1.6	45.8* ±2.6
5	10	AP. Prep. (1) & TI 107(35)	68	5.5**±0.4	8.1**±0.7	9.4 ±1.3	1.6 ±0.2	40.9**±3.1	60.0**±4.8
5	10	TI 107(35)	74	4.2 ±0.4	5.7 ±0.5	2.2 ±0.3	0.6 ±0.1	34.3* ±2.9	46.4* ±3.5
30	10	AP. Prep. (1) & TI 108	74	5.0* ±0.2	6.8* ±0.6	7.5 ±1.3	1.5 ±0.2	36.3 ±3.0	49.1 ±4.5
30	10	TI 108(35)	76	4.0**±0.1	5.3* ±0.2	3.2**±0.7	0.9*±0.2	30.1* ±2.5	39.6**±3.0
300	10	AP. Prep. (1) & TI 109(35)	69	5.3**±0.5	7.7**±0.9	7.8 ±1.5	1.3 ±0.2	31.8 ±3.4	46.1 ±3.6
300	10	TI 109(35)	66	4.2 ±0.2	6.4 ±0.3	2.3* ±0.2	0.5*±0.1	30.3* ±2.5	45.9* ±2.8
		AP. Prep. (1)	68	6.4* ±0.3	9.5* ±1.1	10.2* ±0.7	1.6*±0.1	32.9* ±3.1	48.6* ±2.9
		No Treatment	70	4.3 ±0.3	6.1 ±0.4	1.7 ±0.4	0.4 ±0.1	22.0 ±2.2	31.4 ±2.1

AP. Prep. —Pituitary preparation (Parke, Davis 50 P4) with thyrotrophic and gonadotrophic activity.
TI 102 to TI 105—Pituitary preparation (Armour 264) acetylated at 24° C. The number is merely a code.
TI 106 to TI 109—Pituitary preparation (Parke, Davis 50 P4) acetylated at 1° C. The number is merely a code.
* —Indicates a p value of 0.01 or less.
** —Indicates a p value between 0.05 and 0.01.
†( ) —Indicates total dose in mg. or ml.

at 24° C. A different pituitary preparation (Parke, Davis 50 P4) when reacted with acetic anhydride still retained some ability to increase chick testes weight when the reaction time was progressively increased to 300 minutes at 1° C (Table 3).

With one TSH preparation (Parke, Davis 099861), acetylation for 30 minutes at 27° C gave a more effective inhibitor against exogenous and endogenous TSH than acetylation at 1° C (Table 4).

Volatile acid determination of a pituitary preparation, TI 85, acetylated with acetic anhydride for 30 minutes at 21° C demonstrated 2.45% additional volatile acid groups when compared to the non-acetylated pituitary preparation (Table 5). Assuming a homogeneous preparation with a molecular weight of 10,000 (7), this would then give approximately 5.7 additional volatile acid groups per protein molecule. This corresponded grossly with the difference in amino groups, 6, between the unmodified pituitary preparation and the acetylated pituitary preparation.

DISCUSSION

The ability of acetylated TSH preparations to antagonize the thyroid-stimulating action of pituitary preparations seems to be primarily a property of acetylated TSH preparations. One can not, at present, exclude the possibility that this inhibitory activity is the property of acetylated gonadotrophin, another acetylated protein or another component in an acetylated pituitary preparation. The former is unlikely since inhibition of TSH stimulation occurred with those acetylated pituitary preparations where

TABLE 4. THE RELATIONSHIP OF TEMPERATURE OF ACETYLATION OF PITUITARY PREPARATIONS TO THE STIMULATORY AND INHIBITORY ACTIVITY OF THESE PREPARATIONS

Temp. of acetylation (C)	Type of treatment total dose (mg.)†	No. of chicks	Body wt. (gm.)	Thyroid wt. (mg.)	Thyroid wt./(100 gm. body wt.)	I^{131} uptake		Testes wt. (mg.)	Testes wt./(100 gm. body wt.)
						%/dose	%/mg.		
	AP. Prep. (1.5)	8	57	8.6*±0.9	15.2* ±1.8	15.3* ± 1.3	1.9 ±0.2	33.2*±2.9	58.7* ±6.1
1°	AP. Prep. (1.5) & TI 62(37.5)	10	59	6.2*±0.4	10.4* ±0.5	16.5 ± 2.2	2.6* ±0.3	31.2 ±2.5	52.6 ±4.8
1°	TI 62(37.5)	10	66	3.6*±0.3	5.4* ±0.3	4.4* ± 0.7	1.2**±0.2	27.5*±3.0	41.5* ±3.4
27°	AP. Prep. (1.5) & TI 64(37.5)	8	49	4.6*±0.8	9.4**±1.8	11.4**±16	2.8 ±0.5	27.6*±3.8	57.7 ±4.6
27°	TI 64(37.5)	8	47	2.5*±0.2	5.3* ±0.5	1.4* ± 0.3	0.5* ±0.2	7.3*±1.0	15.1**±1.6
	No Treatment	10	58	4.6 ±0.3	7.9 ±0.4	7.5 ± 0.3	1.6 ±0.2	11.1 ±1.2	19.1 ±1.6

AP. Prep.—Pituitary preparation (Parke, Davis 099861) with thyrotrophic and gonadotrophic activity.
TI 62 —Pituitary preparation (Parke, Davis 099861) acetylated for 30 minutes.
TI 64 —Pituitary preparation (Parke, Davis 099861) acetylated for 30 minutes.
* —Indicates a p value of 0.01 or less.
** —Indicates a p value between 0.05 and 0.01.
†( ) —Indicates total dose in mg. or ml.

the gonadotrophic activity was not completely destroyed. It is apparently not the property of any acetylated protein, since acetylated bovine serum albumin will not inhibit the increase in thyroid weight or increased uptake of radioiodine produced by treating chicks with TSH preparations (Table 2). It is not the property of the acetyl group per se to antagonize TSH, for acetic acid alone has no effect (Table 2). It may be noted that the number of acetyl groups injected with a 1→100 dilution of glacial acetic acid was approximately 50 times the number of acetyl groups introduced with the acetylated pituitary preparation.

These studies give no indication of a pituitary factor which increases thyroid weight being distinct from a factor of the pituitary which affects thyroidal iodine metabolism. In all cases where the acetylated TSH preparations offset the weight response of chicks treated with un unmodified TSH preparation, there was also a reduction in the increased I^{131} uptake in the thyroid noted with TSH treatment alone.

Under no circumstances in this study was it possible to offset the increase in testes weight induced by the injection of a pituitary preparation by the simultaneous administration of an acetylated pituitary preparation, acetylated bovine serum albumin or a 1→100 dilution of glacial acetic acid when the weight was related to total body weight. When pituitary preparations were reacted with acetic anhydride at room temperature, the acetylated product when injected into chicks, resulted in smaller testes than uninjected controls. This was also the case with a 1→100 dilution of

TABLE 5. VOLATILE ACID AND AMINO GROUP ANALYSIS BEFORE AND AFTER ACETYLATION

Preparation	Volatile acid		Amino	
	%	Groups	%	Groups
Pituitary Preparation (Parke, Davis ×6585)	1.29	3.0	0.96	6.0
Acetylated Pituitary Preparation (TI 85)	3.74	8.7	0	0

The calculations are based on the assumption that the pituitary preparation (Parke, Davis ×6585) with thyrotrophic and gonadotrophic activity is homogeneous with a molecular weight of 10,000.

glacial acetic acid, although the number of acetyl groups injected with 1→100 dilution of glacial acetic acid was far in excess of the number of acetyl groups introduced with the acetylated pituitary preparation.

As indicated earlier, studies using S^{35} labeled TSH preparations suggested that thyrotrophin might have both a localizing and a stimulatory property. The latter property apparently could be destroyed and yet the labeled material seemed to retain the localizing property. Assuming this double ability formulation for TSH, one could assume the introduction of acetyl groups as destroying the stimulatory property without affecting the localizing property. More specifically, the free amino groups might be essential for stimulatory activity but not for localization.

The acetylated preparations, which apparently have lost their stimulatory activity, are still able in some cases to prevent the stimulation and presumably the localization of the unmodified thyrotrophin, whether exogenously administered or endogenously produced. If there is a limited number of binding sites in the thyroid available for the localization of TSH, it may be that the excess of acetylated thyrotrophin molecules prevents the localization of unmodified thyrotrophin. Since 4.6 to 35 times as much acetylated TSH as unmodified TSH must be administered to antagonize the effect of the latter, it may be that the acetylated thyrotrophin molecules do not localize in the thyroid gland as efficiently as the unmodified thyrotrophin molecules. There may also be a spectrum of acetylated TSH molecules. Those molecules with a greater number of acetyl groups than the optimum necessary to destroy the stimulatory activity and yet retain the localizing property, possibly might not be included in the greater number of acetylated molecules necessary to offset the stimulation of non-substituted TSH molecules. Perhaps acetylation destroys all the thyroid stimulating activity of a pituitary preparation thus permitting another component, acetylated or unmodified, to act as an inhibitory substance.

The reaction of a protein preparation with a reagent like acetic anhydride is governed by many factors. Determination of the molecular ratio of substituent group to protein is difficult when the protein preparation is a heterogeneous one. Moreover, the number of acetyl groups introduced will be determined by the reaction kinetics of each component protein. The molecular ratio, in such a case, merely represents an average relationship of substituent group to protein. Although the pituitary preparations employed in this study contained gonadotrophins, no attempt was made to characterize these trophic factors as the follicle stimulating hormone, luteinizing hormone or luteotrophic hormone. The molecular weights of these hormones are greater (6) than that assumed for thyrotrophin. It is doubtful whether thyrotrophin has ever been obtained in sufficiently pure form to obtain a reliable molecular weight. Notwithstanding, a molecular weight of 10,000 (7) has been assumed for these pituitary preparations of admitted differing degrees of purity. This value obviously ignores the

higher molecular weights of the gonadotrophins as well as unidentified proteins in the preparations.

Although acetylation of a protein is most often thought to acetylate the alpha and epsilon amino groups, it may well be that other functional groups on the protein are affected. Acetylation in proteins of the phenolic and aliphatic hydroxyl groups as well as sulfhydryl groups has been suggested. This problem has been adequately summarized by Olcott and Fraenkel-Conrat (8). In the present studies, the correspondence between the decrease in free amino groups, and the increase in acetyl groups, would suggest that the amino groups are the major site of introducing this substituent group. Studies in progress may clarify this issue however.

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SUMMARY

Pituitary preparations with thyrotrophic (TSH) and gonadotrophic activity have been acetylated with acetic anhydride. Under all reaction conditions tested, acetylation destroyed the ability of these preparations to increase chick thyroid weight and uptake of radioactive iodine. The destruction of gonadotrophic activity by acetylation was dependent on the temperature and the duration of the reaction. In some cases, these acetylated pituitary preparations were able to offset the increase in thyroid weight and uptake of radioactive iodine produced by an unmodified pituitary preparation. There was no alteration in the gonadotrophic response of chicks treated with a gonadotrophic preparation and the acetylated pituitary preparation. Analysis of the acetylated pituitary preparation revealed complete blocking of the free amino groups with the addition of an appropriate number of volatile acid groups.

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THE EFFECTS OF PURIFIED GONADOTROPHINS ON THE MORPHOLOGY OF THE TESTES AND THUMB PADS OF THE NORMAL AND HYPOPHYSECTOMIZED AUTUMN FROG (*RANA PIPIENS*)[1]

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THE present paper is concerned with the morphological and histochemical changes that are induced in the testes of the normal and hypophysectomized autumn frog by injection of human chorionic gonadotrophin (HCG) and of highly purified mammalian follicle-stimulating hormone (FSH), luteinizing hormone (LH), and luteotrophic hormone (LTH (prolactin)). Observations are also included on the structural changes in the thumb pads which, in the male frog, is a secondary sex character. Two excellent reviews by Houssay (1) and van Oordt (2) have recently appeared which cover the background of the problem and pay particular attention to the effects of gonadotrophic hormone in amphibia.

MATERIALS AND METHODS

Adult male frogs, weighing approximately 40 grams, were procured from a Vermont dealer during the first week of November; they were placed at room temperature (24° C) in community tanks wet with running tap water at 18° C, and were not fed. During the second week of November they were separated into one control group of 5 animals and ten experimental groups of 4 frogs each. Experimental frogs received injections into the dorsal lymphs sac every other day for twenty days (total of 10 injections) of 0.5, 0.05, and 0.005 mg. of LH; 5, 0.5, and 0.05 mg. of FSH; 50 and 5 I.U. of LTH, and 100 and 10 I.U. of HCG.[4] Two to three hours after the last injection, the animals were killed.

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[4] Generous supplies of swine follicle-stimulating hormone, lot #K45308R; sheep luteinizing hormone, lot #227–80; and beef prolactin, lot #R-491153, were kindly provided by Dr. S. Steelman of Armour Laboratories. The degree of purity of this lot # of FSH was determined by bio-assay in hypophysectomized immature rats at the Armour Laboratories and was found to contain 2 to 3% of LH as a contaminant. Its potency was rated at 1 Armour unit/mg. The LH preparation is said to contain traces of FSH and TSH. Human chorionic gonadotrophin (Antuitrin-S, Parke, Davis) was purchased from the hospital pharmacy.

The testes were fixed in Helly's fluid and in Baker's buffered formol-calcium. The testes were dehydrated, embedded in paraffin and sectioned at 3 to 5μ in the routine manner. Sections of the testes fixed in Helly's fluid were stained with hematoxylin and eosin. Other sections were stained by the periodic acid-Schiff (PAS) method, and alternate sections were digested with 1% aqueous malt diastase at 37° C, to remove glycogen before oxidizing and staining them. Blocks of testes fixed in formol-calcium were sectioned with the freezing microtome at 10 to 15μ and stained with sudan black B. Thumb pads were fixed by immersion in buffered 10% neutral formalin and were decalcified in 3% nitric acid; some were embedded in paraffin, sectioned and stained by the PAS method as outlined above, while others were embedded in gelatin, and frozen sections were prepared which were stained with sudan black B.

In order to prove that the observed responses of the testes of normal autumn frogs to FSH and LH were not a result of stimulation by endogenous gonadotrophic secretion, a second experiment was performed in which 35 frogs, procured in early December, were hypophysectomized one day prior to the start of the experiment. These animals were separated into 5 groups of 7 animals each; one control group, 3 groups receiving FSH in the dosages outlined above, and one group receiving a total of 5 mg. of LH, on the same injection schedule and under identical conditions of treatment.[5] Sixteen animals died during the experiment. Three normal frogs were killed at the end of the injection period to serve as controls for the second experiment. Testes from animals in the second experiment were fixed in Helly's fluid and in Stieve's fluid and treated as outlined above. Completeness of hypophysectomy was determined at autopsy by examination of the base of the brain under a dissecting microscope.

Stimulation of spermatogenic proliferation was determined by counting the number of primary spermatogonia in sections of ten seminiferous tubules of each testis. In order to insure some uniformity in area of cells to be counted, the seminiferous tubules were selected so that they filled a high power field (×500). This selection was at random. Additional counts were performed on the testes of the more responsive groups.

OBSERVATIONS

General cytologic events in the spermatogenic cycle of Rana pipiens

The following description, based upon our own study and that of Glass and Rugh (4), summarizes the cytological observations of the testes and thumb pads of Rana pipiens made throughout the annual cycle, in order to provide background for the comparison of these structures with those of hormonally treated frogs. The spermatogenic wave in the frog takes

[5] In the experiment using hypophysectomized frogs, another swine FSH preparation, lot #0178–1, was supplied to us by Dr. J. D. Fisher of the Armour Laboratories. This preparation was adjudged to contain approximately 5% of LH as a contaminant by bio-assay at the Armour Laboratories. Through the courtesy of Dr. M. N. Runner of the Jackson Memorial Laboratory, this preparation was bio-assayed for LH by the induced ovulatory response in immature mice (3). If one assumes that the LH activity as measured by the increase in ventral prostate weight of hypophysectomized immature rats is comparable in degree to the number of ova induced in immature mice which had been primed with 1 I.U. of pregnant mares' serum, then the latter results indicate that appreciably more ovulatory hormone is present in this FSH preparation than the 5% indicated by Armour. In the frog, this difference in LH contamination between the two FSH preparations cannot be detected. Of considerable importance, however, is that the potency of the second FSH preparation is rated at 2 Armour units/mg.

place in the seminiferous tubules within localized clusters of cells that form the "spermatocysts." These formations are in contact with the basement membrane, and are made up of spermatogenic cells surrounded by flattened follicular cells. The first stage of the wave begins with a large cell, the primary spermatogonium, which has a relatively clear cytoplasm and a large, lobulated nucleus which is poor in chromatin and contains prominent nucleoli. Each primary spermatogonium gives rise by mitotic division to many smaller cells, the secondary spermatogonia, which have round or indented nuclei with more abundant chromatin and more basophilic cytoplasm than the primary spermatogonia. The nuclear chromatin of the secondary spermatogonium is gradually transformed into slender filaments that mark the beginning of the prophase of the primary spermatocyte. At this stage, the cells are still large and have round nuclei with one or two prominent nucleoli and less basophilic cytoplasm. At the end of the meiotic division, the newly formed secondary spermatocytes can be recognized by their smaller size, eosinophilic cytoplasm and by the peripheral location of the clumped chromatin of the nucleus. The secondary spermatocytes undergo mitotic division and are transformed into spermatids. In the course of their further differentiation the spermatids elongate and become clustered about the nuclei of the follicular cells. As they mature, the spermatids become greatly elongated and closely packed, with their tips deeply embedded in the follicular cell. At about this stage of development the wall of the spermatocyst becomes discontinuous and the clusters of spermatozoa are exposed to the lumen of the seminiferous tubule. The follicular cell with the spermatozoa cluster now can be recognized as a sustentacular cell or Sertoli cell.

The interstitial tissue of the testes undergoes seasonal changes in morphology. The number and volume of interstitial cells increase gradually in late winter and early spring, reaching maximal development in the breeding season. The cells then diminish in size and number with the advent of summer. A secondary sexual structure, the thumb pad, similarly shows a cyclic behavior; the degree of tumescence and pigmentation of the thumb pad increases in the spring in response to an increased level of circulating androgens, whereas in late summer and autumn with declining testicular activity, the size of the thenar eminence is decreased and the overlying skin is less heavily pigmented.

The cytology of the testes and thumb pads of the normal autumn frog

A) *Untreated frogs.* In control animals the seminiferous tubules are filled with mature spermatozoa and with the early stages of the spermatogenic wave, namely primary and secondary spermatogonia and primary spermatocytes in early prophase (Figs. 1 and 14–18). Sertoli cells contain large droplets of lipide in their basal cytoplasm (Fig. 19) as well as moderate amounts of glycogen (cf. Burgos, 5). The interstitial tissue consists of

many closely packed, fusiform cells (Fig. 10), which contain little or no lipide (Fig. 19). At this time the epithelium of the thumb pads shows a moderate degree of cornification and the subepithelial mucous glands are in an inactive state. Chromatophores are concentrated in a layer immediately beneath the epithelial basement membrane (Figs. 21 and 23).

B) *Frogs injected with luteinizing hormone (LH).* Animals that received LH show striking changes in the histology of the testes and thumb pads. Injection of 0.5 mg. of LH induces a maximal release of spermatozoa (spermiation) from the Sertoli cells and a significant increase in the number of primary spermatogonia (Table 1). The seminiferous tubules appear smaller

TABLE 1. THE RESPONSE OF PRIMARY SPERMATOGONIA IN THE SEMINFEROUS TUBULES OF NORMAL AND HYPOPHYSECTOMIZED AUTUMN FROGS TO THE ADMINISTRATION OF PURIFIED GONADOTROPHINS

Group and total dosage of hormone administered	No. of animals' testes examined	Total no. of tubules counted	Mean no. of primary spermatogonia per ten tubules ±stand. dev.	P value with reference to group controls
Exp. I. Normal autumn frogs				
Controls	3	90	21 ± 6	—
LH-5 mg.	3	70	35 ± 10	<0.01
LH-0.5 mg.	3	40	25 ± 8	>0.3
LH-0.05 mg.	3	70	24 ± 10	>0.4
FSH-50 mg.	2	40	36 ± 11	<0.01
FSH-5 mg.	3	60	37 ± 17	<0.02
FSH-0.5 mg.	3	60	25 ± 12	>0.3
HCG-1000 I.U.	2	20	25	—
HCG-100 I.U.	2	20	29	—
LTH-500 I.U.	2	20	25	—
LTH-50 I.U.	2	20	20	—
Exp. II. Hypophysectomized autumn frogs				
Controls	5	90	25 ± 12	—
LH-5 mg.	3	30	38 ± 8	>0.1
FSH-50 mg.	2	20	52	—
FSH-5 mg.	5	110	49 ± 11	<0.01
FSH-0.5 mg.	4	80	45 ± 10	<0.01

in diameter and contain relatively few clusters of spermatozoa (Figs. 5 and 6). Free spermatozoa in moderate numbers are seen in the lumen of the tubules and in the distended excretory ducts. Long, attenuated Sertoli cells protrude into the lumen, and some appear to be detached from the basement membrane and free in the lumen (Figs. 2, 5 and 7).

The interstitial tissue also undergoes considerable alteration in response to LH at this dosage. The cells are oval or polygonal in shape and have a highly vacuolated cytoplasm and a slightly enlarged nucleus with one or two prominent nucleoli. The intertubular tissue appears more abundant than in the controls (cf. Figs. 2 and 1, and 5 and 1). Inasmuch as no mitotic figures were observed in these cells, it appears that the wider separation of

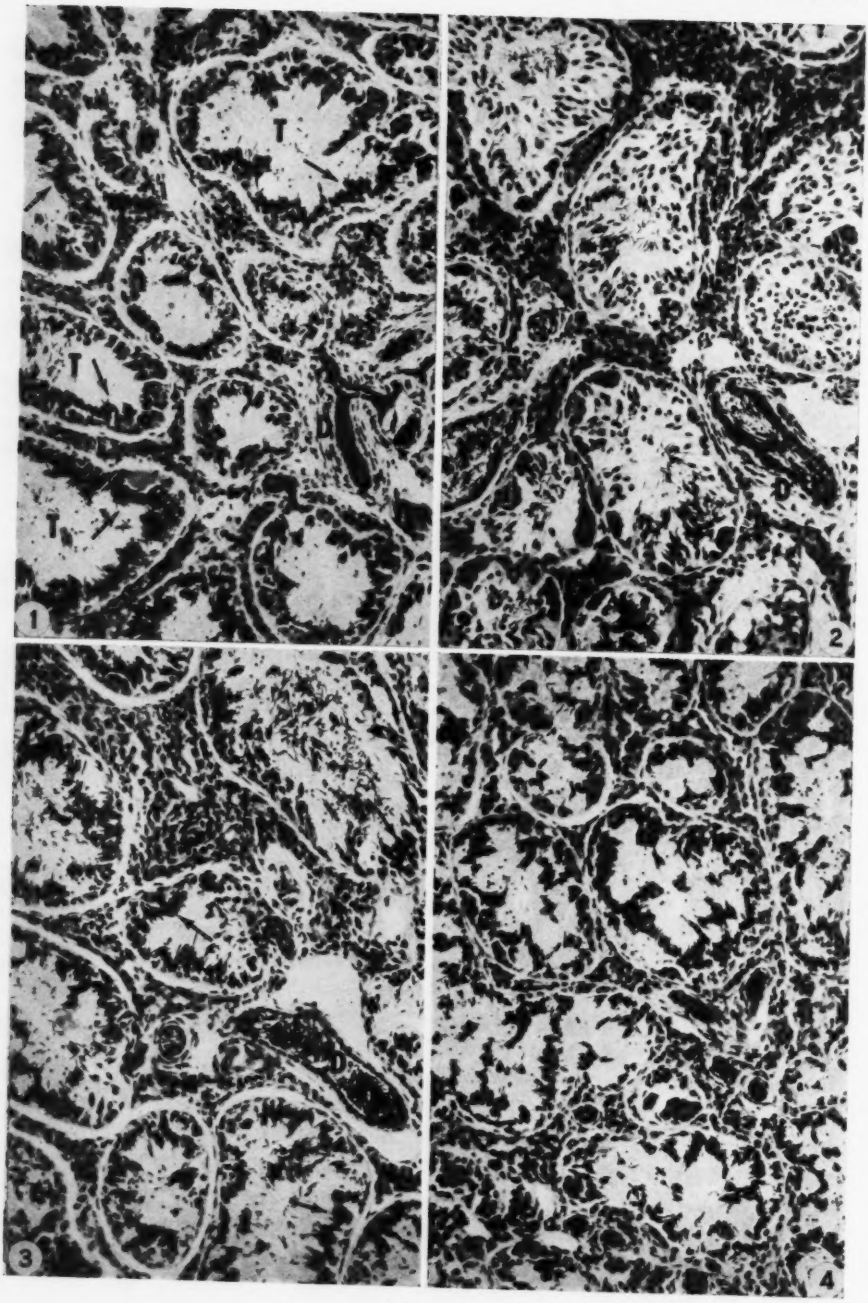

PLATE 1

the tubules may be attributed to the hypertrophy rather than hyperplasia of the interstitial cells. It is clear from the study of sudan-stained sections that the increase in interstitial cell volume is largely due to rapid accumulation of small droplets of stainable lipide after LH administration (Fig. 20). Some lipide is also found in the tubules, where it occurs principally in the Sertoli cells, many of which have separated from the basal membrane and are located within the lumen (Fig. 20). Diastase-digestable, PAS-positive material, presumed to be glycogen, is present in the Sertoli cell cytoplasm (Figs. 7 and 8) in increased amounts after LH injection, especially in those cells which have released the spermatozoa clusters.

Thumb pads of the group of LH-treated frogs are increased in size and more intensely pigmented than those of the controls (Figs. 23, 24). In some areas of the sections, the epithelium is appreciably thicker and more heavily cornified. Melanocytes (chromatophores) have migrated between the epithelial cells (Fig. 24). The mucous glands of the thumb pad show histological evidence of increased activity and possess large nuclei with conspicuous nucleoli. Mucous droplets which are intensely PAS positive are found free within the enlarged lumen of the glands (Fig. 22).

Lower dosages of LH stimulate the testes and thumb pads to a lesser

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EXPLANATION OF FIGURES 1–4

All figures on this plate are of preparations of frog testes stained with hematoxylin and eosin. ×145.

FIG. 1. A section from a testis of a control autumn frog which illustrates the resting state of the testis at this time. Clusters of darkly-staining nuclei (spermatozoa heads) (arrows) are in close contact with the cells lining the seminiferous tubule (T). At the right, a longitudinal section through an efferent duct (D) shows the tall columnar epithelium of its wall, and the absence of spermatozoa in the constricted lumen. Interstitial tissue appears rather evenly distributed between the tubules and ducts.

FIG. 2. A section from a testis of a frog that received ten injections of 0.5 mg. of LH. Most of the spermatozoa and some other cellular elements have been released from the walls of the seminiferous tubules into the lumens of the tubules and into the patent ducts (D). This is the appearance of a maximally stimulated gland. The epithelium of the ducts is flattened in some areas of its course. The interstitial tissue appears to have a greater number of cells with larger round to oval nuclei (see also Figs. 5 and 7).

FIG. 3. A section from a testis of a frog that received ten injections of 0.05 mg. of LH. Some spermatozoa have been released into the lumens of the tubules and ducts, but many clusters of spermatozoa still remain adherent to the walls of the tubule (arrows). This is the appearance of a weakly stimulated gland. The flattened nature of the duct epithelium is shown to good advantage at the right (D). The interstitial tissue appears not to have been stimulated.

FIG. 4. A section from a testis of a frog that also received ten injections of 0.05 mg. of LH, but did not yield a positive spermiation response. The testis looks very similar to that pictured in Figure 1, with respect to all the elements of the gland. The closed ducts have been cut in cross section.

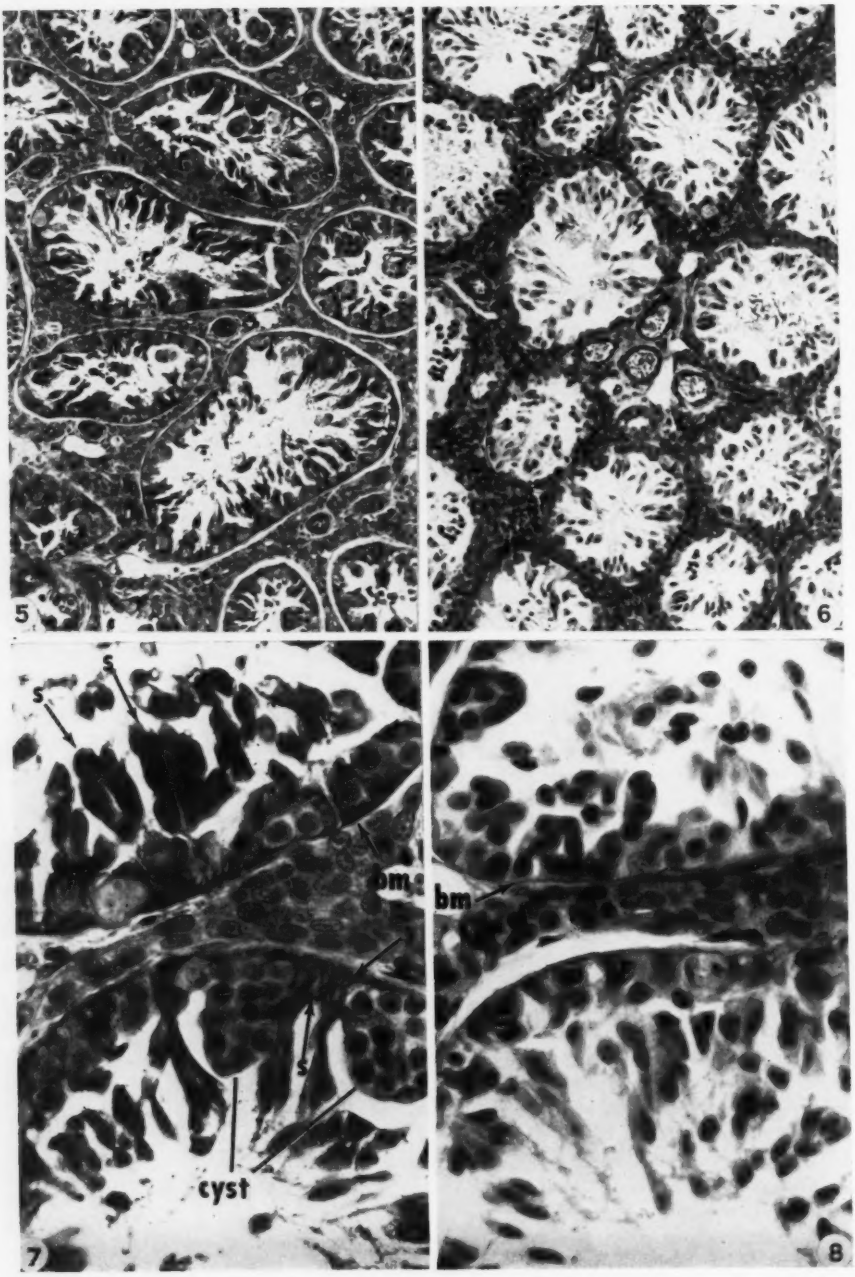

PLATE II

degree. Injection of 0.05 mg. induces a moderate spermiation and a weak interstitial cell response. Glycogen is slightly increased in the Sertoli cells but it appears to be particularly concentrated in the lumens of the tubules. Some stimulation of the thumb pads is suggested by the widening of the lumens of the mucous glands and the enlargement of their cell nuclei.

With a dosage of 0.005 mg. of LH, there is a very weak spermiation response occurring in only a few tubules (Figs. 3 and 4). The interstitial tissue and thumb pads were not stimulated.

C) *Frogs injected with follicle-stimulating hormone (FSH).* Administration of a total dose of 50 mg. of FSH in ten injections of 5 mg. each for twenty days induced a significant increase in the number of primary spermatogonia (Table 1). Glycogen is very abundant in the cytoplasm of the corresponding follicular cells (Figs. 12 and 13), and in the lumen of the tubules. The release of spermatozoa is comparable to that of the group receiving single injections of 0.005 mg. of LH and is due to contamination of the FSH preparation with LH (6, 7).

Interstitial tissue is weakly stimulated, as evidenced by a slight increase in cell volume, the appearance of conspicuous nucleoli and increase in sudanophilia. The thumb pads are also weakly stimulated.

The lower dosage of 0.5 mg. of FSH induced no spermiation at all, but

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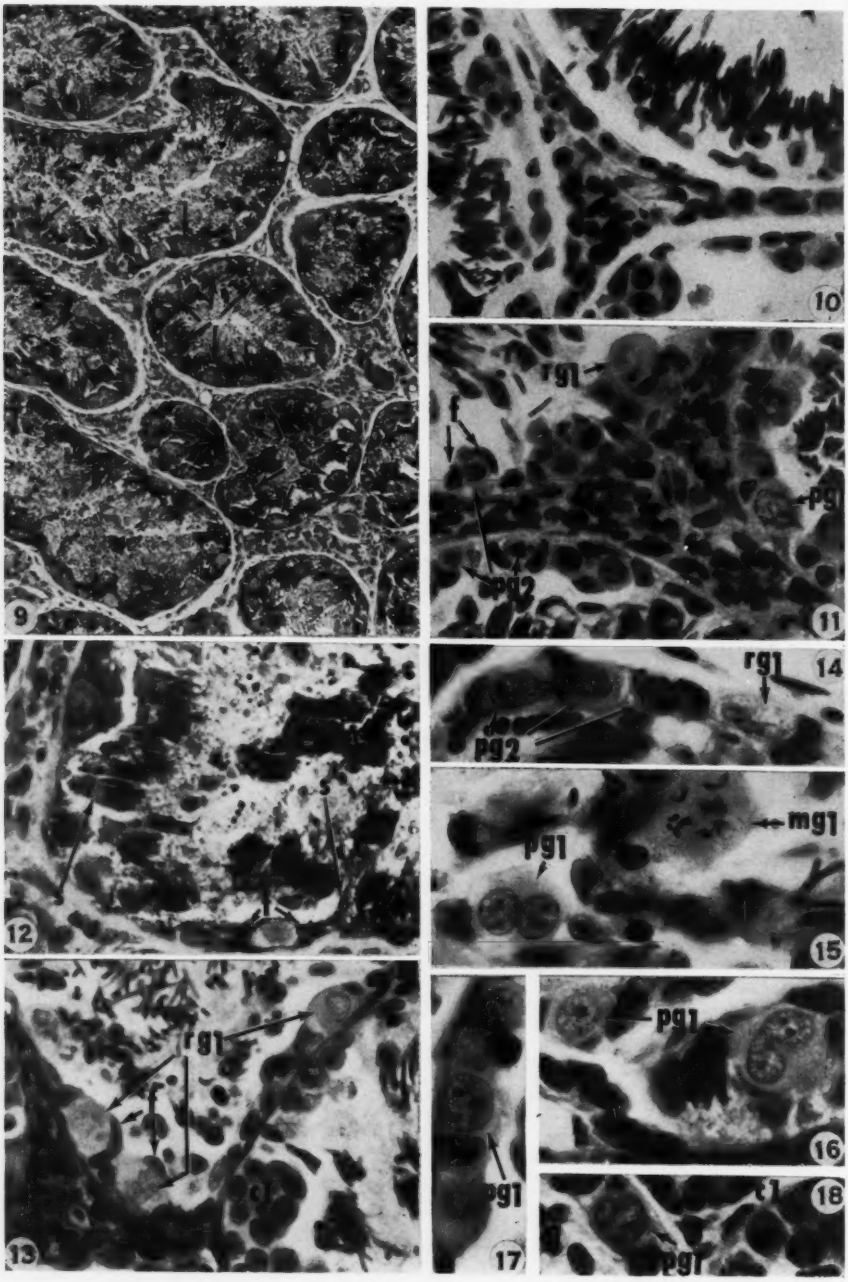
EXPLANATION OF FIGURES 5–8

All figures on this plate are of preparations of frog testis stained with the periodic acid-Schiff (PAS) technique and counterstained with hematoxylin.

FIG. 5. A section of a testis of a frog that received ten injections of 0.5 mg. of LH which shows not only a maximal spermiation response but also a considerable increase in the amount of interstitial tissue. Many cells contain PAS material, which, by comparison with Figure 6, can be ascribed to the presence of glycogen. ×145.

FIG. 6. A similar section to that of Figure 5, which was incubated in a diastase solution prior to staining with PAS. The decrease of staining by some of the cells is due to the elimination of glycogen by enzymatic digestion. The maximal spermiation response is clearly demonstrated in tubules by the striking absence of clusters of spermatozoa. ×145.

FIGS. 7 AND 8. Higher magnification of parts of the sections portrayed in Figures 5 and 6. In Figure 7, note the presence of glycogen which appears as fibrous-like strands (arrows, s, at middle right) in the basal part and as dense condensation in the apical cytoplasm (arrows, s, at upper left) of the Sertoli cells. Figure 8 shows a similar section digested with diastase, prior to staining, which provides evidence that the PAS material seen in Figure 7 is glycogen. On the other hand, the mucoprotein component of the basement membrane (bm) stands out in both figures, being unaffected by the digestion procedure. Two large spermatocysts (cyst) are indicated. Compare the cytological appearance of the interstitial tissue of this preparation with those seen in Figures 10 and 11. The enlarged cells which have prominent chromatin particles disposed within their enlarged nuclei are morphological indications of interstitial cell stimulation. ×500.


9
10
rgl
pgl
pg2
11
12
rgl
14
pg2
mgl
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13
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17
pgl
16
pgl
18

PLATE III

it had a significant effect on the proliferation of primary spermatogonia (Table 1). There was a higher incidence of both primary spermatogonial nuclei with 4 to 6 nucleoli and secondary spermatogonial prophases. Interstitial tissue and thumb pads were unstimulated.

Animals receiving ten injections of 0.05 mg. of FSH showed no signs of stimulation in the testes or thumb pads.

D) *Frogs injected with human chorionic gonadotrophin (HCG).* Ten injections of 100 I.U. of HCG induced spermiation, interstitial stimulation and a weak thumb pad response which was similar to the frogs receiving

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EXPLANATION OF FIGURES 9–18

FIG. 9. A section from a testis of a frog that received ten injections of 0.5 mg. of FSH, which shows the large numbers of spermatocysts conspicuously outlined by the large amount of darkly-staining cytoplasm of the follicular cells. Upon comparison of this photograph with that of Figure 1 of a normal control and also of Figures 2 to 4 of frogs receiving LH treatment, it is obvious that the increase in spermatocyst clusters (arrows) within the tubules is evidence of a stimulatory effect of FSH on the initial stages of spermatogenesis. PAS and hematoxylin. ×145.

FIG. 10. A higher magnification of a section of the testis of a control frog to illustrate the unstimulated condition of the cells of the tubular wall and also of the interstitial tissue. H and E. ×500.

FIG. 11. In contrast to Figure 10, a section of the testis of a frog that received ten injections of 5 mg. of FSH reveals that both spermiation and spermatogenesis have been been stimulated. Note particularly the number of secondary spermatogonia in prophase surrounded by follicular cells (f), one polymorphic nucleus of a resting primary spermatogonium (rg1), as well as a prophase of a primary spermatogonium (pg1). Also compare the apparently weakly stimulated cells of the interstitial tissue with their controls seen in Figure 10, and with those which were treated with LH in Figure 7, in which the interstitial tissue is greatly stimulated. H and E. ×500.

FIGS. 12 AND 13. Sections of the testis of a frog that received ten injections of 0.05 mg. of FSH, which shows, in Figure 12, the distribution of PAS-positive material in some of the cells (f) lining the seminiferous tubules prior to incubation with diastase solution. After incubation with diastase (Fig. 13), PAS staining is abolished in the cytoplasm of the follicular cells (f) and between the heads of the clusters of spermatozoa. Also compare the poor concentration of glycogen in the apical (left arrow, s) and basal (right arrow, s) cytoplasm of the Sertoli cells with that seen in Figure 7, which was stimulated by LH. rg1 indicates the resting state of the primary spermatogonia; c1, primary spermatocytes. PAS and hematoxylin. ×500.

FIGS. 14 TO 18. These are representative regions of the seminiferous epithelium from both control and experimental frogs which show the different appearances of the stages in the maturation of the spermatogonium. The resting nucleus of the primary spermatogonium (rg1) is illustrated in Figure 14 (also see Figs. 11 and 13, rg1). Examples of polynucleolar nuclei of primary spermatogonia in prophase (pg1) are seen in Figures 15, 16, 17, & 18. A primary spermatogonium in metaphase is depicted in Figure 15 (mg1). Secondary spermatogonia in prophase are illustrated in Figure 14 (pg2) (also see Fig. 11). Primary spermatocytes in prophase (c1) are depicted in Figure 18 (also see Fig. 13). All H and E. ×765.

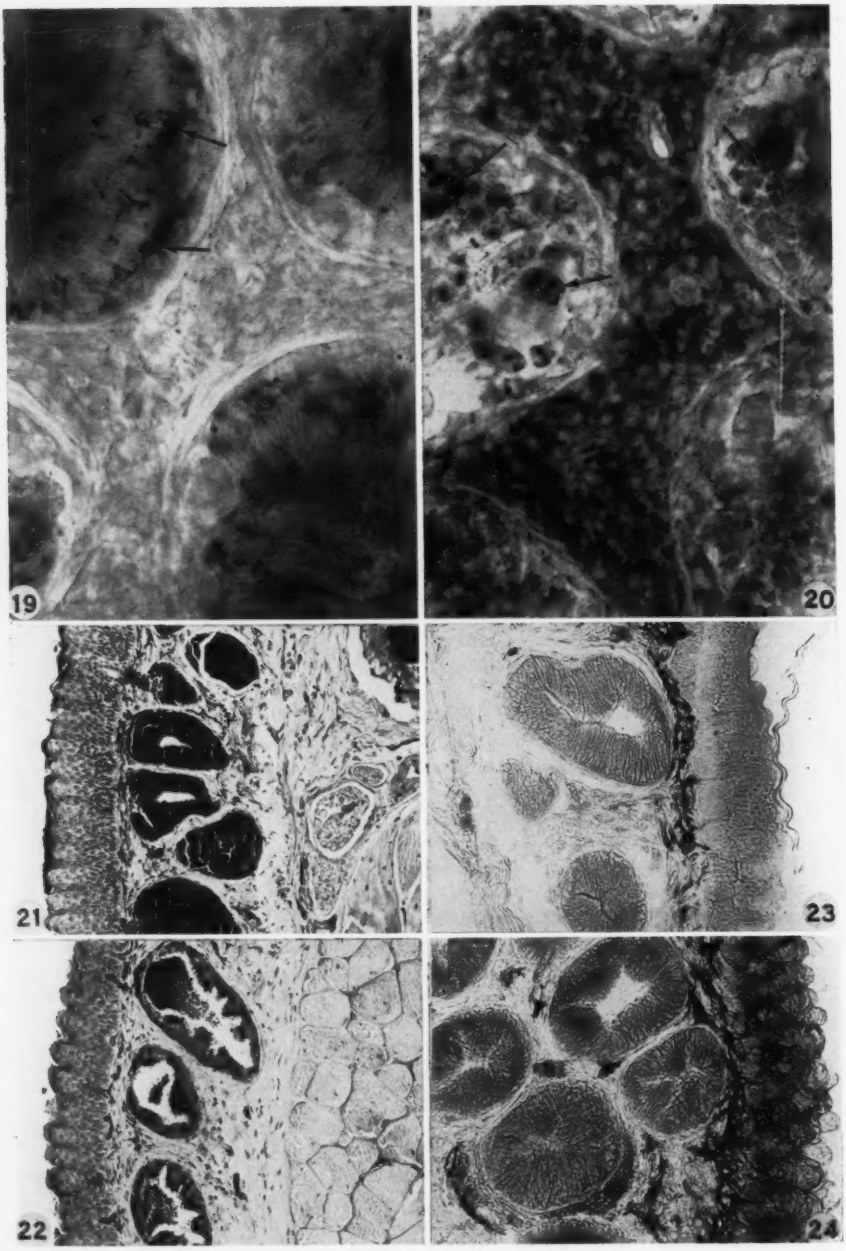

PLATE IV

0.05 mg. of LH in the magnitude of the tissue responses. HCG in a dose of 10 I.U. was ineffective.

E) *Frogs injected with luteotrophin (LTH).* Ten injections of 50 I.U. or 5 I.U. of LTH did not induce morphological changes in any of the tissues examined.

The cytology of the testes of the hypophysectomized autumn frog

The cytology of the testes of control and experimental frogs twenty days after hypophysectomy was similar in most respects to that described above for the normal autumn frog. The increase of diastase-digestable, PAS-positive material in the Sertoli cells after the administration of LH, and in the follicular cells surrounding the spermatogonia or spermatocysts after the administration of FSH, was not as clearly defined in the hypophysectomized frogs as it was in the normal frogs.

The stimulatory effect of FSH and LH on primary spermatogonia is shown in Table 1. Significant increases in the number of primary spermatogonia followed the administration of the lower and medium dosages of FSH and presumably also after the highest dosage, although here, only

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EXPLANATION OF FIGURES 19–24

FIGS. 19 AND 20. Frozen sections stained with sudan black B of the testes of a control frog and of an animal that received ten injections of 0.5 mg. of LH, respectively. In the control, Figure 19, the interstitial tissue is almost devoid of lipide material. Note the parallel arrangement of clusters of spermatozoa heads within the tubules and the large lipide droplets in the basal cytoplasm of the Sertoli cells (arrows). In Figure 20, the interstitial tissue has become filled with a large number of stainable small lipide droplets. Since the testis had been stimulated maximally to evoke a spermiation response, the conspicuous clustering of spermatozoa heads in the tubules is not seen and the lipide droplets contained in the Sertoli cell cytoplasm have now been released into the lumen (arrows). ×315.

FIGS. 21 TO 24. These are photomicrographs of sections of thumb pads from control frogs (Figs. 21 and 23) and from frogs that received ten injections of 0.5 mg. of LH (Figs. 22 and 24). Figures 21 and 22 are paraffin sections stained with PAS and hematoxylin. Figures 23 and 24 are fresh frozen sections stained with sudan black B. All ×85. Figure 21 illustrates the tissue components of the frog's thumb pad and shows the heavily-staining, PAS-positive subepithelial mucous glands in an unstimulated state. Figure 22 shows that, upon stimulation of the interstitial tissue of the testis by the administration of LH, the mucous cells in these glands have hypertrophied and have increased their rate of secretory activity as evidenced by the decreased amount of PAS-positive material in the lumens. Figure 23 shows the faintly staining subepithelial mucous glands and the darker chromatophores in the lamina propria. The epithelium is virtually unstained. Figure 24 reveals that after stimulation of the testes by the administration of LH, there is an increase in the lipide staining in the cytoplasm of the cells of the mucous glands and an apparent migration of the chromatophores between the epithelial cells to effect a darkening of the skin in this region.

two animals survived. Five mg. of LH stimulated spermatogonial proliferation but this was not significant.

DISCUSSION

In the present study, we have endeavored to analyze the morphological changes in the frog testes produced by the administration of purified gonadotrophins by considering three principal activities: 1) spermiation (release of spermatozoa from the Sertoli cells), 2) proliferative effect upon the first stage of spermatogenesis, and 3) interstitial cell stimulation.

Throughout the twenty day treatment period, spermiation could be evoked in frogs receiving adequate amounts of LH or a substance with LH-like activity, HCG. Spermiation responses to the larger dosages of FSH were due to the presence of LH as a contaminant in the preparations (6, 7). Cytologically, the number of spermatozoa present in the seminiferous tubules was found to vary inversely with the intensity of the spermiation.

For the experimental period of 20 days, the morphological responses of the testes of normal and hypophysectomized frogs to the administration of purified hormones were similar. It follows, therefore, that the pituitary of the intact frog is relatively unresponsive to the exogenous hormones, and provides further evidence that the autumn frog, in hibernation, may be regarded as physiologically hypophysectomized.

Although none of the hormones employed was able to stimulate a complete spermatogenic wave, the medium and highest dosages of FSH as well as the highest dosage of LH significantly increased the primary spermatogonia in normal frogs. All three dosage levels of FSH evoked a significant increase in primary spermatogonia in the hypophysectomized frogs. Since the lower dosages of LH had no proliferative effect upon spermatogonia, the LH contamination found in the "purified" FSH can be discounted as the stimulatory agent. On the other hand, since the highest dosage of LH induced a significant increase in the number of primary spermatogonia of normal frogs, the possibility exists that LH in larger quantities may have a proliferative action on spermatogonia. However, it may be more reasonable to suspect that the "trace" contamination of the LH preparation with FSH is responsible for the spermatogenic effects.

This study demonstrates that at least two hormones are necessary to stimulate and maintain testicular function in the frog. FSH in adequate dosages stimulates proliferation of germ cells at the primary spermatogonial stage, whereas sufficient amounts of LH release the last stages of spermatogenesis from the Sertoli cells, and also have an interstitial stimulating effect. Higher dosages of the hormones than those necessary to evoke their principal effects, induce a mixed response which is attributable to the contaminants remaining in the purified hormones.

That glycogen increases in the Sertoli cells in response to the admin-

istration of pituitary glands and diminishes after hypophysectomy has been shown in a previous paper (5). In the present experiment it could be demonstrated that after LH treatment, glycogen was selectively deposited in those Sertoli cells related to spermatozoa clusters, whereas after FSH administration, glycogen was found in the follicular cells which enclose the first stages of spermatogenesis. LH promotes the release of spermatozoa from the apical cytoplasm of Sertoli cells, and both apical and basal portions of these cells have increased amounts of glycogen. On the other hand, FSH stimulates the first stages of spermatogenesis, and the accumulation of glycogen occurs in the cytoplasm of the follicular cells surrounding these elements. In both hypophysectomized and normal frogs the induction of spermiation by the administered hormones reduces the amount of histochemically detectable glycogen in the tubules.

Interstitial cells are stimulated by the higher dosages of LH and HCG. The morphological changes which the interstitial tissue undergoes consist of an increase in cell volume and an accumulation of small lipide droplets. This is in agreement with the previous findings (5) showing that interstitial cells responded similarly in frogs treated with pituitary extracts. The small size of the lipide droplets in the hypertrophied interstitial cells are regarded as indications of secretory activity of cells which produce lipide hormones (8).

Since the secretion of the mucous glands of the thumb pads is dependent upon circulating androgens (9, 10), we have used that response as a further index of stimulation of the interstitial cells of the testis. Only the higher dosages of LH and HCG were able to stimulate the interstitital cells sufficiently to effect noticeable morphological changes in the mucous glands of the thumb pads.

In frogs treated with the lower dosages of FSH, there appeared to be a higher incidence of spermatogonial nuclei containing 4 to 6 nucleoli in a section, compared to the usual one or two found in the controls. If it is assumed that two nucleoli accompany the diploid number of chromosomes, then the fact that the spermatogonia of the FSH-treated frogs contained more nucleoli suggests the possibility of the presence of a polyploid number of chromosomes (11).

A variable finding worthy of mention was the apparent migration of chromatophores between the cells of the epithelium in response to the higher dosages of LH. We have not investigated further whether this phenomenon occurs in response to the direct or indirect action of LH.

SUMMARY

The morphology of the testes and thumb pads of groups of normal and hypophysectomized autumn frogs that received injections of purified gonadotrophins every other day for 20 days was studied. In the testes, three principal activities were analyzed: 1) spermiation (release of sper-

matozoa from the Sertoli cells), 2) proliferative effect upon spermatogenesis, and 3) interstitial cell stimulation. The changes in the histology of the subepithelial mucous glands of the thumb pads provided additional evidence of the secretory activity of the interstitial cells.

The administration of LH or an LH-like substance, HCG (human chorionic gonadotrophin) induced spermiation and interstitial cell stimulation; the magnitude of response was directly proportional to the amount of hormone given. Cytologically, many clusters of spermatozoa were disaggregated and free spermatozoa were found in the lumen of the seminiferous tubules and in the patent excretory ducts. The interstitial cells were increased in cell volume and many small lipide droplets accumulated in the cytoplasm.

The administration of a sufficient dosage of FSH stimulated the early stages of spermatogenesis, as adjudged by the significant increase in the number of primary spermatogonia. Higher dosages of FSH contained enough LH as a contaminant to also induce spermiation.

Prolactin was ineffective in stimulating any of the organs studied.

The specific deposition of glycogen in the follicular cells which surround the first stages of spermatogenesis could be demonstrated after FSH administration. Similarly, after LH administration, glycogen is found in the Sertoli cells related to the spermatozoa clusters.

This study demonstrates that testicular function in the frog is under the control of at least two gonadotrophic hormones: FSH stimulates spermatogenic proliferation and LH induces spermiation and interstitial cell stimulation.

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QUANTITATIVE INVESTIGATION ON THE ANTERIOR PITUITARY-THYROID MECHANISM DURING FROG METAMORPHOSIS

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IN A large number of previous investigations it has been definitely proved that in amphibians metamorphosis is dependent upon the normal functioning of the anterior pituitary-thyroid system (with regard to the extensive literature, see 1, 2). Little is known, however, regarding the variations in thyroid function during metamorphosis and the corresponding changes in the thyrotropic activity of the anterior pituitary, and the statement commonly found in textbooks that thyroid activity increases during metamorphosis is based chiefly on morphological studies. Furthermore, the time relations between the supposed functional variations and between these and the various stages of metamorphosis have been subjected to little investigation. For this reason an attempt has been made to study the functional variations possibly occurring in the anterior pituitary-thyroid system during the various stages of metamorphosis using a quantitative method.

MATERIAL AND METHODS

The species used in this investigation was the South African clawed toad, *Xenopus laevis* Daudin, reared in our laboratory. The animals were reared under standardized conditions and by standardized methods which have proved suitable for this species (3, 4). Under these conditions, the tadpole stage lasts 8 weeks. In what follows, the developmental stage of the tadpoles is indicated only as age. For a more accurate notion of the corresponding stage of development, the reader is referred to the table of normal development for this species (4). Using mainly the terminology suggested by Etkin (2, 5), the material may be classified as follows:

1. Premetamorphic period, 2nd–4th week. 2. Prometamorphosis, 4th–6th week. 3. Metamorphic climax, 6th–8th week. 4. Postmetamorphic period, 8th–12th week. 5. Adults, 2 years.

The animals included in the developmental series were investigated at one-week intervals. A total of 236 animals were studied by the following methods:

a. The thyroid gland was fixed for 24 hours in Bouin's solution and serially sectioned in paraffin at 7 μ. The staining was Heidenhain's azan. The relative amount of the different tissue components was determined by linear measurement (6). Using 1000× projection magnification, three lines, 20 cm. each, were measured from three sections of both glands from three animals at each stage of development.

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b. When the thyroid area and the brain had been dissected out, the remaining parts of the animals were homogenized to an even mass, from which PBI was chemically determined after dilution (7). The determinations, made in duplicate, are based on the mass obtained from at least six animals.

c. Prior to sacrifice, some animals of each stage of development were kept for 96 hours in a radioactive iodine solution (200 μC. carrier-free iodine-131 in 1000 cc. of water), the temperature of which was the same as that of the aquarium water (+22° C). The animals were not fed during the experiment. These animals were then killed, and ground in a glass homogenizer after dissection of the thyroid area and the brain. The proteins were precipitated from the homogenized mass, and the precipitate was washed. Subsequently the thyroid area and the protein precipitate were counted in a scintillation counter, calibrated against a normal iodine solution. A minimum of 6 animals from each developmental stage were investigated. (Since the animals varied greatly in size during development, the iodine content of the thyroid gland is indicated in the following per unit fresh weight of the animal.)

d. The brains were fixed for 6 hours in Helly's solution, rinsed for 12 hours in running water, and mounted in paraffin after passage through a butanol series. The brains were serially sectioned at 4 μ in the transverse plane. For staining, two modifications of the periodic acid-Schiff method were used: Trichrome—PAS (8) and methyl blue—PAS (9). Furthermore, control stainings with Heidenhain's azan were performed after formol—alcohol fixation.

e. The nuclei of the cells containing PAS-positive granules were drawn with a camera lucida, using a magnification of 2200×. In these drawings the area of the nucleus was measured with a planimeter. From three animals of each group a total of 300 nuclei were measured.

RESULTS

All quantitative results are demonstrated in Table 1. Furthermore, the functional variations observed in the anterior pituitary-thyroid system are presented in Figure 1, in which the different curves are not, however, quantitatively comparable. The changes found in the thyroid gland are shown in Figures 2-5. In the histological picture of the anterior pituitary, definite changes were observable during development:

At the age of 2 weeks, definitely PAS-positive granules were clearly

TABLE 1. DETERMINATIONS RELATING TO THE ACTIVITY OF THE ANTERIOR PITUITARY THYROID SYSTEM DURING THE DEVELOPMENT OF *Xenopus laevis*

Age, wks.	Weight, mg.	I^{131} in the thyroid gland, 10^{-3} μC. /100 mg.	PBI^{131} in the organism, 10^{-3} μC. /100 mg.	PBI in the organism, 10^{-3} μg. /100 mg.	Epithelium percentage	Colloid percentage	Size of the nuclei of the PAS+ cells, u^2
2	11	29	34				
3	24	36	51	+			
4	106	80	88	2.04			23.7
5	231	260	95	2.15	57.8	27.7	32.3
6	650	587	63	2.35	67.5	24.3	33.7
7	955	321	35	4.50	90.8	5.7	31.5
8	770	97	13	6.10	67.7	26.0	22.6
9	833	415	73	4.80	50.0	42.9	20.0
10	900	472	62	5.40			
12	1515	176	13	4.10	45.2	45.9	21.3

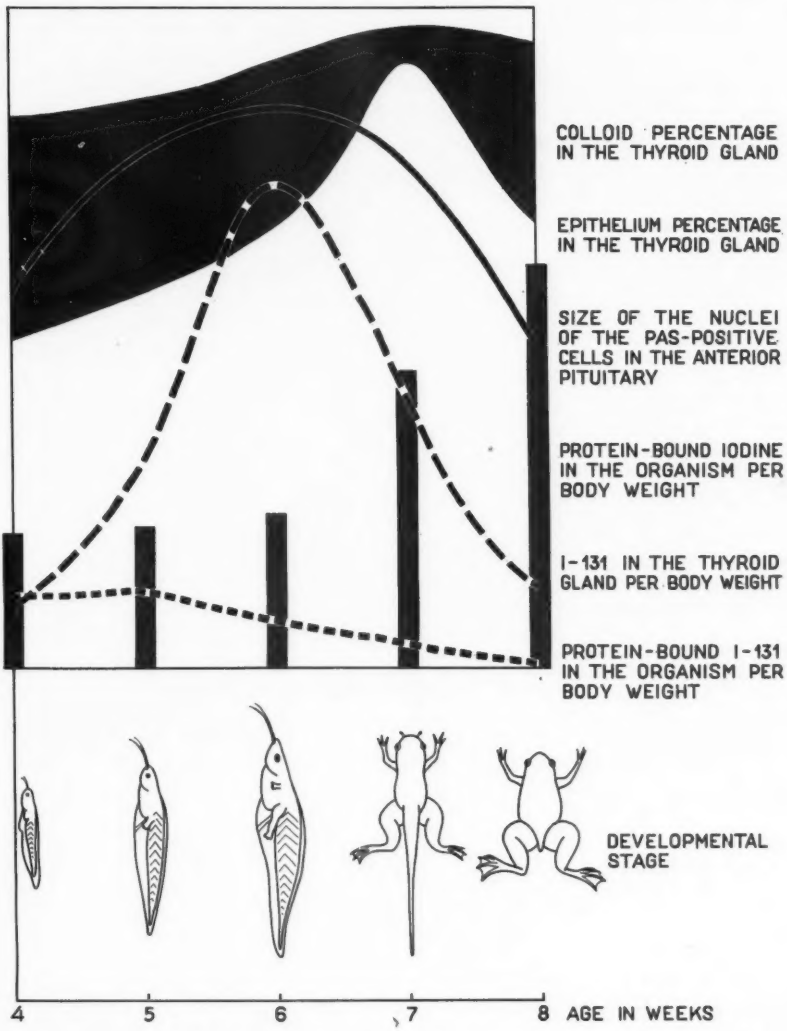
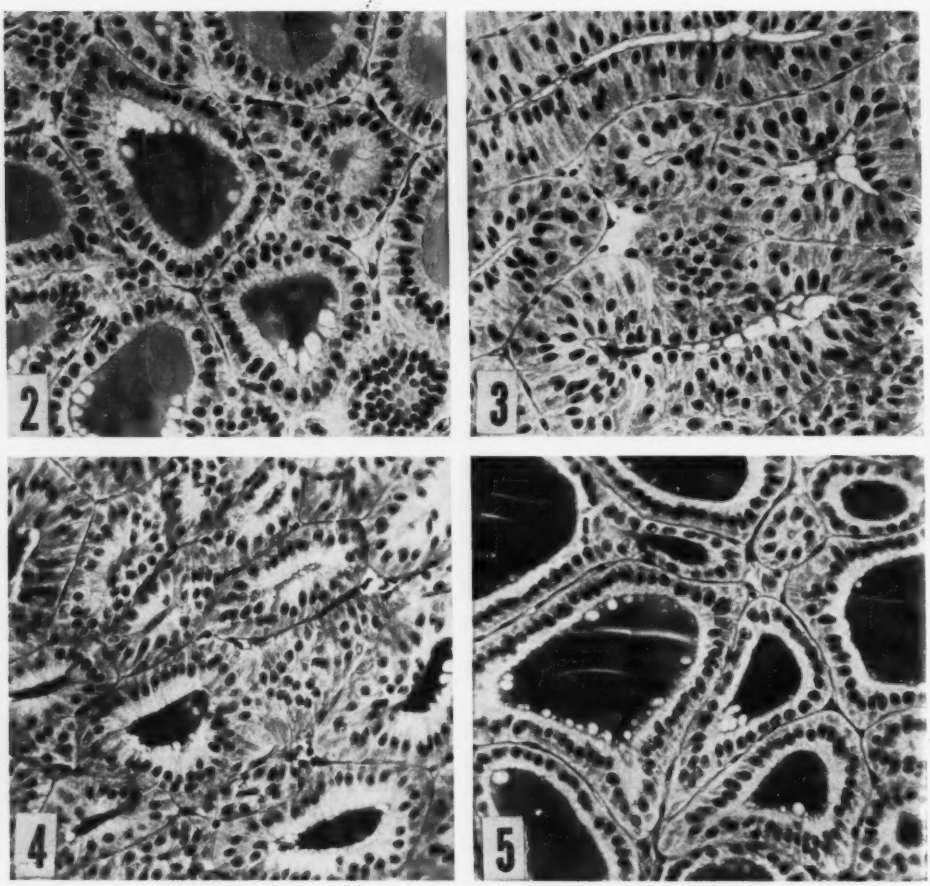

COLLOID PERCENTAGE IN THE THYROID GLAND
EPITHELIUM PERCENTAGE IN THE THYROID GLAND
SIZE OF THE NUCLEI OF THE PAS-POSITIVE CELLS IN THE ANTERIOR PITUITARY
PROTEIN-BOUND IODINE IN THE ORGANISM PER BODY WEIGHT
I-131 IN THE THYROID GLAND PER BODY WEIGHT
PROTEIN-BOUND I-131 IN THE ORGANISM PER BODY WEIGHT
DEVELOPMENTAL STAGE
4
5
6
7
8
AGE IN WEEKS

FIG. 1. The changes in the anterior pituitary-thyroid system quantitatively established during the development of *Xenopus laevis* tadpoles.

distinguishable in some cells in the anterior pituitary. During the subsequent two weeks these became arranged in a firm islet on the ventral surface of the gland. At least until the age of 4 weeks the cells in question were relatively small, and their granulation was irregular. During prometamorphosis the nuclei increased in size, and the cytoplasm and the PAS-positive granulation increased. At the time of the metamorphic climax, the typical ventral arrangement of the PAS-positive cells was broken up and the

latter became dispersed over the whole area of the anterior pituitary. The cytoplasm remained abundant, but its granulation decreased markedly. As a result of this degranulation, sparsely granulated and completely granule-free "chromophobes" were encountered at the end of metamorphosis and immediately afterwards. Four weeks after metamorphosis such cells were no longer discernible, the PAS-positive granulation being again more abundant.

At 2 weeks, the acidophilic cells had also differentiated. Initially the granulation of these cells, too, increased, but by as early as the 5th week it began to decrease. At 7 weeks the acidophils were almost degranulated, large, acidophilic globules being only occasionally encountered in them. Immediately following this stage the granulation of the acidophils in-



FIGS. 2–5. Microphotographs showing the histology of the thyroid gland of *Xenopus laevis* aged 6, 7, 8 and 9 weeks. Heidenhain's azan. Photo: Obj. 20×, oc. 8×.

creased again, and at the end of metamorphosis clearly acidophilic cells were present in abundance.

In adult animals three different types of cell were distinguished in the anterior pituitary with the methyl blue—PAS technique: acidophils, PAS-red cells and PAS-purple cells (Fig. 6). In the present tadpole ma-

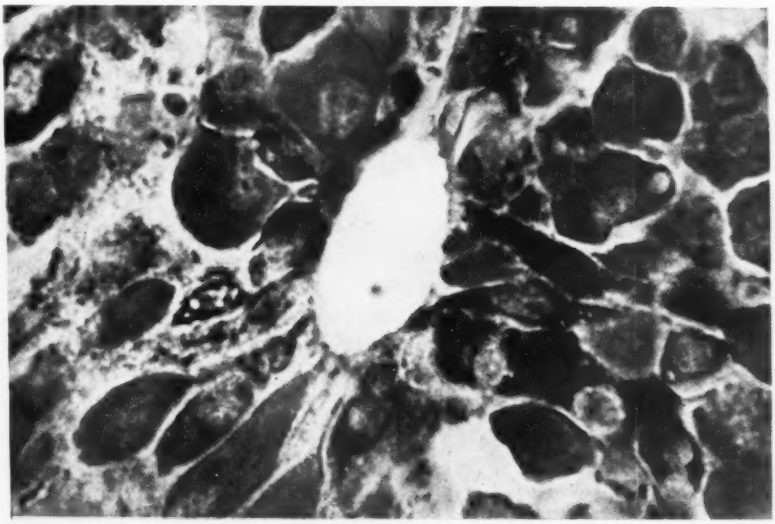


FIG. 6. A microphotograph showing the three types of cells in the anterior pituitary of *Xenopus laevis*. Acidophils (centre and lower left), PAS-purple "gonadotrophs" (upper left) and PAS-red "thyrotrophs" (upper and centre right). PAS-methyl blue stain (9). Photo: Obj. 90× immers., oc. 8× komp. Ektachrom.

terial, the last-mentioned type could not be observed, even though the same staining was employed and the slides were stained simultaneously.

The present results may be summarized as follows:

1. The premetamorphic period. ThI^{131} (*i.e.* I^{131} in the thyroid gland) and both PBI^{131} and the total PBI in the tissues remaining after dissection of thyroid and brain were low. The total PBI was just at the limit of sensitivity of the method of determination. The two types of the anterior pituitary cell, the acidophils and the PAS-positive cells, had differentiated.

2. Prometamorphosis. ThI^{131} rose considerably, and PBI^{131} showed an initial increase, followed by a slow decrease. The total PBI remained the same. In the ratio epithelium/colloid no definite changes were yet dis-

cernible. The granulation of the PAS-positive cells increased, and the nuclei of these cells increased in size.

3. The metamorphic climax. ThI^{131} dropped considerably, and PBI^{131} also decreased considerably, although more slowly. The total PBI rose to three times the previous value. The follicles collapsed, and the amount of colloid decreased abruptly. The PAS-positive cells became degranulated, and their nuclei decreased in size.

4. The postmetamorphic period. ThI^{131} and PBI^{131} rose again immediately after metamorphosis, only to drop after 3–4 weeks. PBI also gradually decreased. Definite histological changes were no longer observable in the thyroid gland. The PAS-positive granulation in the anterior pituitary increased again, but in regard of nuclear size no changes occurred.

DISCUSSION

In brief, the results relating to iodine metabolism may be interpreted as follows: It is obvious that thyroid function definitely commences before the onset of metamorphosis, although the rate of iodine metabolism is still relatively low. During prometamorphosis the iodine uptake rises considerably, but the unchanged PBI together with the gradually decreasing PBI^{131} show that the release of iodine has not increased. The process taking place is apparently an increased storage of iodine in the thyroid gland. At the time of the climax this stored iodine is released, as appears from the decrease in colloid, the decrease in the amount of iodine retained by the thyroid gland, and the increase in the total protein-bound iodine. The simultaneously occurring decrease in PBI^{131} may be due to the fact that the iodine released by the thyroid gland has, in the main, been stored before the beginning of the 4-day experiment, so that the increased secretion is not reflected in the values obtained for the indicator iodine. At the same time the results seem to indicate that the iodine uptake at the end of metamorphosis is lower than during the storage phase, and that the thyroid gland is in a phase in which secretion predominates. After metamorphosis, a transient increase is again observable in the radioactive iodine in the thyroid and in the body. It seems reasonable to infer that the maximal release occurring at the end of metamorphosis is followed by a compensatory phase, persisting for a few weeks.

In contrast to the findings made in previous investigations with radioactive iodine (10), the present results may be regarded as establishing that during metamorphosis definite changes in thyroid function are observable. The results confirm the commonly accepted view that the activity of the thyroid gland increases towards the end of metamorphosis. This view is based on a large number of previous morphological studies of the thyroid gland, summarized by Allen (11) as follows: "First there has been an accumulation of colloid during the early development of hindlegs, followed

by active colloid discharge resulting in the rapid body changes of metamorphosis." The view that the activity of the thyroid increases gradually was also advocated by Etkin (12). Investigating the effect of thyroxine dilutions of different concentrations on the metamorphosis of thyroidectomized tadpoles, he found that only a gradually increasing concentration of thyroxine was capable of inducing normal metamorphosis. The present results seem, however, to indicate that increased thyroid activity is conditional only for the changes occurring during the climax of metamorphosis, whereas during the whole prometamorphic period no increase in the hormonal secretion of this gland was observed. This view is also substantiated by a comparison of the values in question with the results obtained on spontaneously neotenic tadpoles (13) and on tadpoles congenitally lacking a thyroid gland (14).

In the attempt to evaluate, on the basis of the present results, the changes in the thyrotropic activity of the anterior pituitary occurring during metamorphosis, two criteria are possible: Firstly, the changes in the activity of the target organ, *i.e.* the thyroid gland, described in the foregoing, and secondly, the histological changes observable in the anterior pituitary.

In investigations on chicks, Wahlberg (15) found that simple TSH stimulation brought about an increase in the relative amount of epithelium after 2 hours, an increase in the iodine release after 24 hours, and a rise in the iodine uptake after 48 hours. The same events, although clearly delayed, were observed in the present investigation during the 6th–9th weeks. The "delay" is no doubt due to the fact that the process here involved is not a momentary TSH stimulation, but a gradually increasing release of thyrotropin, to which the target organ reacted by gradual changes in activity. Thus, adopting this criterion, it may be assumed that the secretion of TSH does not increase until the time of the climax, *i.e.* during the 7th week of development. The fact that no increase was observed in the relative amount of epithelium or in the iodine release during the earlier developmental stages seems to constitute evidence against an increased TSH release during this time. Consequently it may, perhaps, be assumed that the storing or adaptive phase of thyroid activity occurring prior to the climax is at least in part autonomous and independent of the control of the anterior pituitary. The experimental results of VanderLaan (16) also seem to indicate that of the phases of thyroid function, storage is to some extent autonomous. On the basis of the foregoing it thus seems reasonable to conclude that during prometamorphosis an increase in hormonal synthesis, to be regarded as a preparation for the hyperfunction occurring during the climax, occurs both in the thyroid gland and in the anterior pituitary, and that these events are at least in part independent of each other.

Since convincing evidence has been produced for the view that the

thyrotropic function of the anterior pituitary is associated with its PAS-positive cells (8, 17, 18, 19, 3, 20), interest is mainly focussed on these in connection with the problems here concerned. According to Pearse (17), the PAS-positive granulation signifies that the cells in question contain the mucoprotein hormones TSH, FSH and LH. On the basis of certain earlier experimental and histological investigations, however, it seems obvious that the gonadotropic function of the anterior pituitary of the anurans does not commence until metamorphosis is completed (21, 22, 20). With certain reservations, the gonadotropins may thus be excluded from this discussion, it being assumed that the PAS-positive granulation in the anterior pituitary of the present tadpole material signifies the presence of thyrotropin. Attention will be centered on two morphological criteria of the functional state of the PAS-positive cells, *viz.* nuclear size and the relative amount of granulation. It may be assumed that the former is a measure of the activity of the cells, being a "funktionelles Kernödem" (23), whilst the latter reflects the relationship between hormone synthesis and release (*e.g.* 24, 25). According to this interpretation, the activity of the PAS-positive cells increases during prometamorphosis, which, together with the simultaneously occurring increase in granulation, seems to be indicative of intensified hormonal synthesis. The continued increase in granulation, and the activity observable in the target organ, seem to indicate that the secretion, on the other hand, has not yet increased. At the beginning of the climax, functional changes were observed in the target organ, which were interpreted above as evidence of an increased release of TSH. Simultaneously a decrease in granulation, obviously signifying increased secretion, was observed in the PAS-positive cells of the anterior pituitary. The simultaneously occurring decrease in nuclear size may be interpreted as a sign of decreased hormonal synthesis, the hormone released having been synthesized and stored previously, *i.e.* during prometamorphosis. (In the foregoing it was established in regard of thyroid function that the corresponding storage phase changed into a secretive phase, and that the hormone released had in this case, too, been stored earlier in development.) The decreased synthesis and increased release during the final stage of metamorphosis result in complete degranulation of the cells, such as has previously been described after thyroidectomy (26, 27) and in connection with thyroid aplasia (14). That the thyrotropin stores of the anterior pituitary become exhausted after metamorphosis is also proved by the transplantation experiments performed by Etkin (28). When transplanting the hypophyses of tadpoles of various developmental stages into premetamorphic tadpoles, this investigator found that the capacity of the transplants to accelerate metamorphosis decreased in the final stage of metamorphosis. A rise in the thyrotropin level of the anterior pituitary during development has also been demonstrated in transplantation experiments (29, 30).

CONCLUSIONS AND SUMMARY

The following points were investigated on *Xenopus laevis* tadpoles of various developmental stages: a) The relative amount of epithelium and colloid in the thyroid gland. b) The amount of protein-bound iodine in the body. c) The iodine metabolism in the experimental animals, using radioactive iodine. d) The histology of the anterior pituitary, with special reference to the PAS-positive granulation. e) The size of the nuclei of the PAS-positive cells.

On the basis of the results obtained, the following conclusions are drawn with regard to the functional variations in the anterior pituitary-thyroid system: In the premetamorphic phase of low activity the function of the anterior pituitary-thyroid system has commenced, but no definite changes are yet discernible.

In the prometamorphic storage phase preparation for future hypersecretion occurs both in the anterior pituitary and in the thyroid. (In both glands this is manifested as increased hormonal synthesis and storage.)

An increased hormonal secretion is observable in both glands, at the beginning of the climax and persists until the end of metamorphosis. The onset of this phase is obviously preceded by an increase in the thyrotropic secretion of the anterior pituitary, resulting in increased thyroid secretion and a rise in the protein-bound iodine in the body. At the end of metamorphosis and immediately afterwards maximal evacuation is observed in both glands.

Postmetamorphically a compensatory phase occurs in thyroid function, and persists for some 3–4 weeks.

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EFFECT OF CHLORPROMAZINE ON STRESS INDUCED ADRENAL ASCORBIC ACID DEPLETION[1]

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WE HAVE previously summarized our results on the effects of Dibenzyline, adrenal cortex extract, and morphine on stress induced adrenal ascorbic acid depletion (1). Since various reports (2–7) on the effects of chlorpromazine on pituitary-adrenal function have been somewhat conflicting, we have extended our investigations to include this agent.

METHODS

White male rats, descendants of the Wistar strain, weighing 150–200 gm., were used for all experiments. In all except one experiment the rats were anesthetized with sodium pentobarbital intraperitoneally (4.0–5.0 mg. per 100 gm.).

The stresses applied to induce adrenal ascorbic acid depletion were sham adrenalectomy, unilateral adrenalectomy, l-epinephrine injected subcutaneously, and vasopressin injected intravenously. In each case the time from the onset of stress, or injection of corticotropin, to termination of the experiment was one hour.

Chlorpromazine[2] (dissolved in physiological saline) was injected subcutaneously. In testing for the effects of chlorpromazine per se the experiment was terminated one hour after injection. In testing for the ability of chlorpromazine to inhibit stress induced adrenal ascorbic acid depletion, the drug was injected one hour prior to the onset of stress. In testing for the ability of chlorpromazine to inhibit the effects of exogenous corticotropin, the drug was injected one hour prior to the corticotropin.

Adrenal ascorbic acid depletion was used as an index of ACTH secretion (8). In intact rats depletion was estimated by comparing adrenal ascorbic acid concentrations in each experimental group with the concentrations in a simultaneous control group injected with comparable volumes of physiological saline. In hypophysectomized rats[3] the left adrenal was first removed as a control for each rat. Ascorbic acid was determined by the chemical method of Roe and Kuether (9).

RESULTS

As indicated in Table 1, chlorpromazine produced no significant depletion of adrenal ascorbic acid over a dosage range of 0.25 to 2.0 mg. per 100

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[1] The preliminary results of this investigation were presented at the Thirty-Eighth Annual Meeting of the Endocrine Society, Chicago, 1956 (*J. Clin. Endocrinol. and Metab.* **16:** 915. 1956).

[2] Kindly supplied as "Thorazine" by the Research and Development Division of Smith, Kline and French Laboratories, Philadelphia 1, Pa.

[3] Obtained from Hormone Assay Laboratories, Chicago, Ill.

TABLE 1. EFFECT OF CHLORPROMAZINE ON ADRENAL ASCORBIC ACID CONCENTRATION IN ANESTHETIZED RATS

Exper. No.	Group	No. of rats	Procedure*	Adrenal ascorbic acid concentration (mg./100 gm.)		P (Fisher's t table) compared with group a
				Mean	S.E.	
1	a	13	Control-Saline	459	17.0	
	b	12	Chlorpromazine-0.25 mg.	500	15.8	>0.05
2	a	8	Control-Saline	472	20.9	
	b	11	Chlorpromazine-0.50 mg.	472	26.3	>0.50
3	a	10	Control-Saline	503	29.9	
	b	13	Chlorpromazine-0.50 mg.	528	29.7	>0.50
4	a	13	Control-Saline	385	20.4	
	b	11	Chlorpromazine-1.0 mg.	337	15.0	>0.05
5	a	11	Control-Saline	508	11.1	
	b	12	Chlorpromazine-1.5 mg.	466	16.5	>0.05
6	a	10	Control-Saline	448	19.5	
	b	10	Chlorpromazine-2.0 mg.	405	14.1	>0.05

* Doses per 100 gm. body weight s.c.

gm. body weight, in rats anesthetized with sodium pentobarbital. Similarly, the largest dose attempted (2.0 mg./100 gm.) also did not produce depletion in unanesthetized rats (Table 2). These were all single injections of chlorpromazine; no prior "adapting" or training procedures were used.

Table 3 shows the effect of chlorpromazine on the adrenal ascorbic acid depletion produced by sham adrenalectomy. As indicated, chlorpromazine in doses of 1.5 or 2.0 mg. per 100 gm. body weight effectively prevented adrenal ascorbic acid depletion, whereas doses of 0.25 to 1.0 mg. per 100 gm. were ineffective. As shown in Table 4 the largest dose of chlorpromazine (2.0 mg./100 gm.) also prevented the adrenal ascorbic acid depletion produced by unilateral adrenalectomy. In view of the small number of animals used and the large standard errors, the failure of the 1.5 mg. dose of chlorpromazine to prevent depletion is not necessarily indicative of a larger dose requirement against this stress versus that against sham adrenalectomy.

TABLE 2. EFFECT OF CHLORPROMAZINE ON ADRENAL ASCORBIC ACID CONCENTRATION IN UNANESTHETIZED RATS

Exper. No.	Group	No. of rats	Procedure*	Adrenal ascorbic acid concentration (mg./100 gm.)		P (Fisher's t table compared with group a
				Mean	S.E.	
1	a	16	Control-Saline	361	15.8	
	b	16	Chlorpromazine-2.0 mg.	351	12.5	>0.50

* Doses per 100 gm. body weight s.c.

TABLE 3. EFFECT OF CHLORPROMAZINE ON ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY SHAM ADRENALECTOMY

Exper. No.	Group	No. of rats	Procedure*	Adrenal ascorbic acid concentration (mg./100 gm.)		P (Fisher's t table)	
				Mean	S.E.	Compared with group a	Compared with group b
1	a	13	Control-Saline	459	17.0	—	—
	b	13	Sham Adrenalectomy	371	6.1	<0.01	—
	c	11	Chlorpromazine-0.25 mg. +Sham Adrenalectomy	399	17.8	<0.05	>0.50
2	a	10	Control-Saline	437	17.1	—	—
	b	10	Sham Adrenalectomy	309	18.1	<0.01	—
	c	9	Chlorpromazine-0.5 mg. +Sham Adrenalectomy	365	28.0	<0.05	>0.10
3	a	11	Control-Saline	494	20.7	—	—
	b	9	Sham Adrenalectomy	339	21.6	<0.01	—
	c	13	Chlorpromazine-1.0 mg. +Sham Adrenalectomy	331	23.1	<0.01	>0.50
4	a	11	Control-Saline	508	11.1	—	—
	b	10	Sham Adrenalectomy	377	16.9	<0.01	—
	c	10	Chlorpromazine-1.5 mg. +Sham Adrenalectomy	483	21.5	>0.30	<0.01
5	a	10	Control-Saline	336	13.2	—	—
	b	10	Sham Adrenalectomy	244	7.8	<0.01	—
	c	10	Chlorpromazine-2.0 mg. +Sham Adrenalectomy	353	28.2	>0.50	<0.01

* Doses per 100 gm. body weight s.c.

The effectiveness of chlorpromazine (2.0 mg./100 gm.) in preventing the depletion produced by a large dose of l-epinephrine is shown in Table 5 (Experiment 1). On the other hand, there was no indication that chlorpromazine (2.0 mg./100 gm.) has the ability to inhibit vasopressin induced adrenal ascorbic acid depletion (Table 5, Experiment 2). It might be argued

TABLE 4. EFFECT OF CHLORPROMAZINE ON ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY UNILATERAL ADRENALECTOMY

Exper. No.	Group	No. of rats	Procedure*	Adrenal ascorbic acid concentration (mg./100 gm.)		P (Fisher's t table)	
				Mean	S.E.	Compared with group a	Compared with group b
1	a	8	Control-Saline	377	20.2	—	—
	b	7	Unilateral Adrenalectomy	275	27.0	<0.01	—
	c	6	Chlorpromazine-1.5 mg. +Unilateral Adrenalectomy	308	40.3	>0.10	>0.4
2	a	10	Control-Saline	336	13.2	—	—
	b	10	Unilateral Adrenalectomy	237	8.7	<0.01	—
	c	10	Chlorpromazine-2.0 mg. +Unilateral Adrenalectomy	324	29.8	>0.50	<0.02

* Doses per 100 gm. body weight s.c.

TABLE 5. EFFECT OF CHLORPROMAZINE ON ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY EPINEPHRINE AND BY VASOPRESSIN

Exper. No.	Group	No. of rats	Procedure*	Adrenal ascorbic acid concentration (mg./100 gm.)		P (Fisher's t table)	
				Mean	S.E.	Compared with group a	Compared with group b
1	a	12	Control-Saline	342	11.2	—	—
	b	12	l-epinephrine-0.06 mg.	275	10.5	<0.01	—
	c	12	Chlorpromazine-2.0 mg. +l-epinephrine-0.06 mg.	372	13.1	>0.50	<0.01
2	a	10	Control-Saline	448	19.5	—	—
	b	10	Vasopressin-0.1 unit i.v.	323	25.7	<0.01	—
	c	8	Chlorpormazine-2.0 mg. +Vasopressin-0.1 unit i.v.	301	21.6	<0.01	>0.50

* Doses per 100 gm. body weight (s.c. except where specified).

that the intensity of the vasopressin stress was too severe to permit the inhibitory action of chlorpromazine to become manifest. We do not believe this to be the case for the following reasons: 1) The maximal stress induced depletion obtainable is, in our hands, approximately 200 mg. per 100 gm., or to about 50% of the control level. Doses of vasopressin of 0.07 to 0.1 unit per 100 gm. body weight have consistently produced less depletion than these maximal values. 2) Sham adrenalectomy, the effect of which is blocked by chlorpromazine (1.5–2.0 mg./100 gm.), produced an average depletion of adrenal ascorbic acid to 70.9% (56.5–80.8) of the control level in 15 experiments; vasopressin (0.1 unit/100 gm.) produced an average depletion to 72.4% (61.9–77.8) in four experiments. 3) There was no indication of even partial inhibition of vasopressin induced adrenal ascorbic acid depletion by chlorpromazine (Table 5, Experiment 2).

Table 6 shows that chlorpromazine (1.5 mg./100 gm.) is not effective in preventing adrenal ascorbic acid depletion produced in hypophysec-

TABLE 6. EFFECT OF CHLORPROMAZINE ON ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY EXOGENOUS ACTH IN HYPOPHYSECTOMIZED RATS

Exper. No.	Group	No. of rats	Procedure*	Adrenal ascorbic acid depletion† (mg./100 gm.)		P (Fisher's t table)
				Mean	S.E.	Compared with group a
1	a	7	ACTH 3.0 μg. i.v.	−155	19.8	
	b	6	Chlorpromazine 1.5 mg. s.c. +ACTH 3.0 μg. i.v.	−196	16.6	>0.1
2	a	7	ACTH 10.0 μg. i.v.	−202	25.8	
	b	6	Chlorpromazine 1.5 mg. s.c. +ACTH 10.0 μg. i.v.	−234	11.2	>0.3

* Doses per 100 gm. body weight.
† Left adrenal (control)-right adrenal, ascorbic acid concentration.

tomized rats by exogenous corticotropin (3.0 or 10.0 μg./100 gm.). It is to be recalled that this same dose of chlorpromazine almost completely prevented the effect of sham adrenalectomy; the latter stress (Table 3, Experiment 4) produced a degree of depletion (−131 mg./100 gm.) similar in magnitude to that produced by the smallest dose of corticotropin (−155 mg./100 gm.).

DISCUSSION

For convenience, our own results concerning the inhibitory effects of various agents on stress induced ACTH secretion (as indicated by adrenal ascorbic acid depletion) are summarized schematically in Table 7. The inhibitory action of chlorpromazine against the various stresses we have applied is similar to that of adrenal cortex extract; we do not infer, however, that this gross similarity indicates a similar mechanism or even a similar site of action. In our hands morphine remains the only agent capable of inhibiting the effect of vasopressin as well as the effects of all other stresses applied. We have already expressed our views (1) concerning the interpretation of the above, and similar, data reported previously. In the absence of knowledge concerning the specific sites of these inhibitory actions, the data obtained with chlorpromazine and with morphine suggest central nervous system (possibly hypothalamic) sites of action. Allowing for possible future deficiencies in such an assumption, the data appear at the present time to lend support to hypotheses implicating a central neural or neurohumoral mechanism for the regulation of ACTH secretion.

TABLE 7. SUMMARY: INHIBITION OF STRESS INDUCED ACTH SECRETION IN ANESTHETIZED RATS

Stress	Inhibition* of ACTH Secretion			
	Dibenzyline†	Adrenal cortex extract†	Chorpromazine	Morphine†
Adrenergic amines	+	+	+	+
Sham adrenalectomy	0	+	+	+
Unilateral adrenalectomy	0	+	+	+
Vasopressin	0	0	0	+

* Significant inhibition indicated by + sign.
† Results reported previously (Ref. 1.)

The results of the present investigation confirm the results obtained by Olling and de Wied (4), demonstrating that chlorpromazine significantly inhibits adrenal ascorbic acid depletion induced by unilateral adrenalectomy or by epinephrine. These investigators also found inhibition of the effects of histamine and noradrenaline. Their results are also similar to ours in that chlorpromazine did not inhibit the effect of exogenous ACTH (0.25 μg./100 gm.). However, they state that chlorpromazine given sub-

cutaneously in unanesthetized rats caused adrenal ascorbic acid depletion; our results demonstrate no difference between saline injected and chlorpromazine injected unanesthetized rats. Other workers have also shown inhibition by chlorpromazine of adrenal ascorbic acid depletion induced by laparotomy (5), unilateral adrenalectomy (2), and injection of formalin (3); effective doses in these studies varied markedly among investigators (1.0–10.0 mg./100 gm.). On the other hand, Holzbauer and Vogt (6) failed to demonstrate inhibition by chlorpromazine (1.0 mg./100 gm.) of the adrenal ascorbic acid depletion induced by surgical stress or injection of adrenaline. In addition Nasmyth (7) found that chlorpromazine in doses of 0.25 mg. per 100 gm. failed to inhibit the effects of surgical stress, epinephrine or large doses of histamine. Some of these investigators (5, 6, 7) also found that chlorpromazine itself, in doses of 1.0–10.0 mg. per 100 gm., caused adrenal ascorbic acid depletion in unanesthetized rats.

Some of the above discrepancies seem to us assignable to one or more of the following differences in technique: 1) Judging from our own results with sham adrenalectomy, doses of chlorpromazine less than 1.0–1.5 mg./100 gm. might well fail to show any apparent inhibitory action against moderate to severe stress, while larger doses may do so. 2) Doses of chlorpromazine sufficient to inhibit the effects of moderate to severe stress in animals anesthetized with sodium pentobarbital, may not be adequate in unanesthetized animals or in animals anesthetized with other anesthetics (ether, urethane). 3) A dose sufficient to inhibit stress induced adrenal ascorbic acid depletion in animals anesthetized with sodium pentobarbital may at times complicate a study in unanesthetized animals by producing some depletion itself. Our results indicate, however, that chlorpromazine (2.0 mg./100 gm.) in *untrained* unanesthetized rats produces no greater effect in *1 hour* than does saline—a comparison not evident in some of the above reports. On the other hand Holzbauer and Vogt (6) found that chlorpromazine (1.0 mg./100 gm.) did produce some depletion in trained rats in *4½ hours* when compared to saline. Thus the time interval here may be important; we have shown previously that Dibenzyline i.v. causes no depletion in 1 hour but does cause depletion in 24 hours (10). 4) The onset and duration of this inhibitory action of chlorpromazine has not been adequately defined. Whether or not the inhibitory action manifest at 1 hour will be apparent at shorter or longer intervals after injection is uncertain. Intervals as short as 30 minutes were used in some of the above studies and as long as 20 hours in others.

Less work has been done on the effects of chlorpromazine on pituitary-adrenal function using blood or urinary adrenal corticoids as an index. Vandewater and Gordon (11) found no indication of inhibition of operative stress in humans by chlorpromazine in doses of 50–175 mg.; they used urinary corticoids as an index of pituitary-adrenal responses to stress. Egdahl and Richards (12) found that chlorpromazine (1.3–10.0 mg./kg.

i.v.) increased adrenal venous 17-hydroxycorticosteroids in unanesthetized dogs (adrenal veins previously cannulated under anesthesia) and in dogs anesthetized with sodium pentobarbital; this effect of chlorpromazine was prevented by hypophysectomy. Harwood (13) also observed that chlorpromazine (5.0–10.0 mg./kg.) caused an increase in 17-hydroxysteroids in peripheral blood in unanesthetized monkeys. On the other hand, the stress of insulin hypoglycemia in humans has been reported to be inhibited by pre-treatment with 100–400 mg. of chlorpromazine (14); blood 17-hydroxysteroids were used as an index. In the same study pretreatment with chlorpromazine did not prevent the adrenal response to exogenous corticotropin. Thus even these more direct methods have, as yet, not particularly clarified the actions of this drug on pituitary-adrenal function.

SUMMARY

In this study chlorpromazine injected subcutaneously did not cause adrenal ascorbic acid depletion in either anesthetized or unanesthetized rats, when compared with saline as a control. Adrenal ascorbic acid depletion produced by sham adrenalectomy, unilateral adrenalectomy, or by epinephrine was effectively inhibited by prior administration of chlorpromazine; depletion produced by vasopressin was unaffected by chlorpromazine. Adrenal ascorbic acid depletion produced by exogenous corticotropin in hypophysectomized rats was also unaffected by chlorpromazine. The inhibitory effects of chlorpromazine on stress induced adrenal ascorbic acid depletion are discussed with reference to other inhibitory agents (Dibenzyline, adrenal cortex extract, morphine). An attempt was made to explain some of the discrepancies among various reports relating to this problem.

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THYROID WEIGHT, MICROSCOPIC STRUCTURE, AND RADIOIODE CONCENTRATION AT VARIOUS LEVELS OF THYROTROPHIN SECRETION IN THE GOITROGEN-TREATED RAT[1]

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IN HYPOPHYSECTOMIZED rats, propylthiouracil has been shown to potentiate the response of the thyroid iodide trap to thyrotrophic stimulation (1). The question of whether or not a similar potentiation of this response occurs in intact rats is of importance in interpreting the fact that, in rats with anterior hypothalamic lesions, there is an apparent dichotomy between the metabolic and growth responses of the thyroid gland to goitrogen treatment (2, 3, 4). This question has been examined by Greer (3), who studied thyroid-serum radioiodide concentration (T/S) ratios and thyroid weights in goitrogen treated intact rats (100 gm. in weight) maintained at three different levels of thyrotrophin secretion. Secretion of this hormone was regulated by daily injections of thyroxine, according to the method of Dempsey and Astwood (5).

He found that whereas the T/S and thyroid weight were approximately normal in rats given 3.5 or 5.0 μg. doses of racemic thyroxine daily, both were elevated in rats maintained on 2.5 μg./day. He concluded from this that, in the absence of hypothalamic damage, the T/S and thyroid weight respond similarly to endogenous thyrotrophic stimulation, despite the presence of goitrogen.

However, since the study did not include animals treated with less than 2.5 μg./day of thyroxine, this conclusion cannot be applied beyond the observed dose range. The present report deals with an extension of Greer's experiment over a wider range of thyroxine dosage. Histologic observations on the thyroid glands of the test animals have been included.

MATERIALS AND METHODS

The entire experiment was carried out on groups of young female, Holtzman rats, which were given large doses of propylthiouracil[2] (PTU) and graded doses of L-thyroxine[3]

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[2] Propylthiouracil was generously provided by Drs. R. J. Turner and R. W. Cunningham of the American Cyanamid Company.

[3] The L-thyroxine pentahydrate used in the first experiment was kindly provided by

concurrently for 10 days. Three separate trials were carried out, using two basic experimental designs:

I. Thirty-eight rats, 140–215 gm. in weight, bred in this laboratory from Holtzman stock, were divided into five groups with mean body weights between 159 and 163 gm. All were given PTU in Purina fox chow (0.15%), and tap water, *ad libitum*. In addition, the rats in four of the groups were injected intraperitoneally every 12 hours with an alkaline (pH 8–9) saline solution of thyroxine (0.5 mg.%), prepared daily from an alkaline stock solution which was kept refrigerated in the dark throughout the 10 day period. Injection volumes were calculated to provide a 160 gm. rat with either 1, 2, 3 or 4 μg. of thyroxine/100 gm./day.

IIa. Forty-two rats, weighing between 107 and 136 gm., were purchased from Holtzman and divided into five groups (mean body weights, 118–123 gm.). Each rat was injected subcutaneously once a day with 0.6 cc. of an aqueous 2 gm.% PTU suspension. Thyroxine was administered as before, in appropriate volumes of a 0.3 mg.% solution, providing either 1, 2, 3 or 4 μg./100 gm./day for a 120 gm. rat. All groups were given Purina fox chow and tap water, *ad libitum*, and their daily food consumption was estimated.

IIb. Forty-one additional animals (weighing 104–123 gm.), divided into four groups with mean body weights between 112 and 117 gm., were subjected to the treatments used in trial IIa. The 4 μg. thyroxine dosage was omitted, and the rats which did not receive thyroxine were injected intraperitoneally twice daily with 0.8 cc. of alkaline (pH 8–9) saline. Food consumption was not recorded in this trial.

T/S determinations were carried out[4] as described by VanderLaan and Greer (6), 12–15 hours after the last thyroxine injection. In cases where an entire thyroid lobe constituted too large a specimen for convenient counting, a 6–10 mg. portion thereof, weighed to the nearest 0.1 mg., was used. One lobe of each thyroid gland was fixed for histologic study. In each of the three trials, 5–6 glands at each dose level were examined microscopically.

Statistical analyses employed Student's *t* test.

RESULTS

The thyroid weight and T/S data from Experiment I, in which PTU was fed, are presented in Figure 1. As thyroxine dosage was increased from 0 to 1 μg., thyroid weight decreased markedly. It continued to decrease between 1 and 2 μg., but was not influenced by changes above the 2 μg. dose level. By contrast, the T/S response curve remained elevated between the 0 and 2 μg. thyroxine doses but dropped rapidly thereafter.

The data from trials IIa and IIb, in which the goitrogen was given by injection, have been pooled for presentation in Figure 1. Although the thyroid weight and T/S values for the rats which were injected with PTU and alkaline saline (trial IIb) were about 10% lower than for those given PTU alone (trial IIa), the differences were not statistically significant. The

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Dr. Harry Fevold of Travenol Laboratories, Inc. The stock solutions used in the second experiment were prepared from a supply of the same drug which was generously donated by Dr. Joseph Harris of the Department of Biochemistry, Albany Medical College.

[4] We are happy to acknowledge the courtesy of Dr. Donald H. Baxter in extending to us the facilities of the Radioisotope Laboratory, Albany Medical Center.

findings at the 1, 2 and 3 μg. thyroxine dose levels coincided almost exactly in the two trials.

The thyroid weight response curve in Experiment II (a and b), although it was irregular between 0 and 1 μg., was essentially similar to that in experiment I. The T/S ratio again remained elevated over the lower thy-

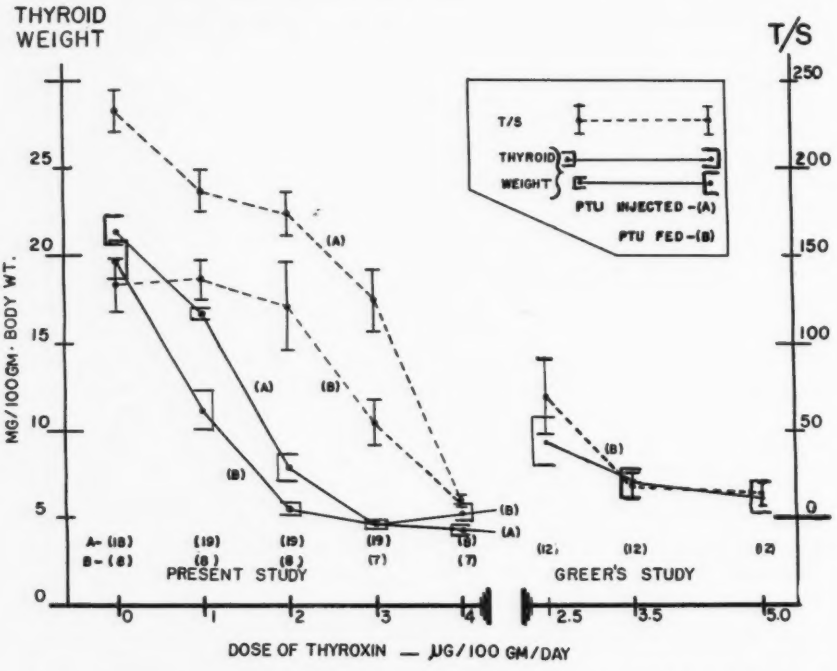

FIG. 1. Thyroid weights and T/S ratios in Experiment I (curves labeled B on left side of graph) and Experiment II (curves labeled A) of the present study, and in Greer's (3) study (curves labeled B on right side of graph), at various doses of thyroxine. Levels of thyrotrophin secretion (inversely proportional to thyroxine dosage) increase from right to left. All rats were given large doses of propylthiouracil (PTU). Letters A and B indicate mode of PTU administration (see box). Parentheses near dosage scale contain the number of rats used in determining the data for each dose in each experiment. Vertical lines through, or brackets around, points on graphs signify standard errors. L-thyroxine was used in this study; Greer used racemic thyroxine.

roxine dose range. However, it dropped somewhat between 0 and 1 μg. (233 ± 12.4 to 187 ± 12.1, $p = .014$). Between 1 and 2 μg. the curve appeared almost flat, but between 2 and 3 μg. it resumed its descent, reaching a hypophysectomy-like value (10.4 ± 2.9) at the 4 μg. dosage.

The following histological descriptions of the thyroid glands refer to the central follicles. In Experiment I, at the 3 and 4 μg. thyroxine dose levels

(slight thyrotropic stimulation) these follicles uniformly contained dense colloid and were lined with squamous epithelium. Some of the glands at the 2 μg. level exhibited cuboidal epithelium and vacuolated colloid, whereas others appeared less active. The difference between 2 and 1 μg. was striking. At the 1 μg. dosage, the follicles contained only traces of colloid, the epithelial cells were tall columnar, and many mitotic figures were evident. The glands of the rats which were not given thyroxine were histologically indistinguishable from these.

In Experiments IIa and IIb, the thyroid histologic responses to thyrotrophic stimulation were similar to those described for Experiment I. At the lower levels of stimulation (3 and 4 μg. of thyroxine) the colloid appeared quite dense and the acinar cells were squamous. The nuclei seemed practically co-extensive with the cell boundaries at 4 μg., whereas at 3 μg. the cells were somewhat larger and more cytoplasm could be discerned. The response at the 2 μg. thyroxine dosage was considerably greater than it was in Experiment I. Here, in every case studied, the colloid was vacuolated and the epithelium was either cuboidal or low columnar. In the glands with columnar epithelium, mitotic figures could be seen. At the two highest levels of thyrotrophic stimulation, the thyroid glands appeared alike, having tall columnar epithelium, with many mitoses, somewhat folded in upon acini which were virtually devoid of colloid.

Thus, in both experiments, the thyroid glands of rats maintained on the 2 μg. thyroxine dosage could be distinguished quite readily, as a group, from those of the 1 and 3 μg. dose groups. In Experiment II the 3 μg. dose group tended to exhibit histologic evidence of greater thyroid activity than the 4 μg. group. Histologically, the two highest levels of stimulation could not be distinguished from each other in either experiment, although they were readily separable on the basis of thyroid weight. Thyroid histology seems to offer a somewhat more sensitive measure of low levels of thyrotrophic stimulation—i.e., while colloid discharge and cellular hypertrophy constitute the predominant structural responses—than does the measurement of thyroid weight. With higher levels of stimulation, however, hyperplasia becomes the predominant histological thyroid response, and the extent of thyroid hyperplasia is more easily assessed by weighing the gland than by microscopic techniques.

The rats in Experiment I did not eat well, and most of them lost weight. However, in Experiment II food consumption was high and all groups gained weight. In trial IIa, where it was measured daily for each group, control food consumption averaged 15 gm./rat/day. The thyroxine treated rats ate more (up to 25 gm./rat/day), intake being roughly in direct proportion to thyroxine dosage. Weight gains, however, which were between 20 and 34 gm./rat/10 days (group averages), were greatest in the 2 μg. groups in both parts of Experiment II.

DISCUSSION

This study clearly demonstrates that the T/S may respond more abruptly than thyroid weight to augmentation of thyrotrophin secretion. In intact, goitrogen treated rats there is a level of thyrotrophic stimulation (permitted here by the administration of approximately 2 μg. of L-thyroxine/100 gm./day) at which the T/S and thyroid weight are as widely separated as in goitrogen treated rats with hypothalamic lesions (2, 3, 4). An examination of Greer's (3) data, plotted here in Figure 1, suggests that the dichotomy would have become apparent if only slightly lower thyroxine doses had been used.

A comparable digression of the T/S response curve from that followed by thyroid weight, as thyrotrophic stimulation is varied, has recently been demonstrated by Halmi and Stuelke (7). These authors have pointed out that the T/S is "not always a reliable measure of iodide pump activity." The iodide trapping capacity of the thyroid gland is a function of its size, as well as of the iodide concentration ratio between thyroid tissue and serum (T/S). Accordingly, the assertion (3) that hypothalamic lesions fail to depress the metabolic activity of the thyroid gland seems unwarranted. The 15 mg. gland of a rat with a hypothalamic lesion, although it may concentrate serum iodide two hundred-fold, does not trap as much iodide as the 40 mg. gland of an unoperated control which also exhibits a 200:1 T/S ratio.

The considerable differences between the T/S response curves, and the less marked difference between the thyroid weight curves, in the first and second experiments of the present study, require comment. In Experiment II, both thyroid weight and T/S were greater at the 1 and 2 μg. dose levels than they were in Experiment I, and the T/S was greater at the 3 μg. level. Although this might be attributable to the better nutrition and/or younger age of the rats used, or to the different mode of PTU administration, a more probable explanation is suggested by the irregularity, in Experiment II, of the weight response curve between the 0 and 1 μg. thyroxine dosages. This flattening of the (usually exponential-like) curve, in both trials IIa and IIb, suggests that the sample of thyroxine used in these trials may have been somewhat less potent than the original. However, it should be pointed out that even if the actual doses of this drug were less than they were intended to be, the relationship between them (volumetrically determined) was nonetheless strict, and both thyroid weight curves were quite regular above the 1 μg. level. If a correction were to be made for this presumed thyroxine dosage error in Experiment II, the initial fall of the T/S curve between 0 and 1 μg., giving it a sigmoid character, would be accentuated. This fall was not found in Experiment I. To compound the discrepancy, Halmi and Stuelke (7) have found that, in PTU

pretreated rats, increasing thyrotrophin secretion actually lowers the T/S at this end of the response curve.

To explain the irregularity of the T/S response to variations in the degree of thyrotrophic stimulation, Halmi (8) has suggested that weak or initial thyrotrophic stimulation may act to increase the number of iodide acceptor sites in the thyroid gland. These sites would seem to approach a maximum tissue concentration rapidly, so that further increases in their absolute number, in response to stronger or longer thyrotrophic stimulation, would require the formation of new tissue. Unless the addition or activation of new acceptor sites proceeded commensurately with the addition of new tissue, the concentration of such sites in the gland as a whole would drop, and the T/S would actually decrease. Such a stepwise progression of events, complicated by the influences of factors other than thyrotrophin which are believed (7) to affect the iodide trap, seems to provide a reasonable explanation for some of the variations in the curves under consideration.

Whether or not this explanation proves to be true, it is evident, at least in goitrogen-treated rats, that the range over which the T/S has a direct relationship to the degree of thyrotrophic stimulation is limited to levels which scarcely, if at all, affect thyroid weight. Within this range, however, the T/S ratio may provide an exquisitely sensitive index of the degree of stimulation. It appears to be far more sensitive than histological criteria, such as cell height and colloid storage, which are most reliable in a range intermediate between the steep portion of the T/S curve and the steep portion of the exponential-like weight response curve. Thus, it would appear that none of these parameters of thyroid activity, by itself, is as useful in assessing the level of thyrotrophic stimulation as is the combination of all three.

SUMMARY

Thyroid weight, radioiodide concentration (TS ratio), and microscopic structure were studied in goitrogen treated rats which were maintained at several levels of thyrotrophin secretion by graded doses of thyroxine. The T/S responded to very slight thyrotrophic stimulation, and achieved virtually maximal values with levels of secretion which failed to affect thyroid structure greatly, if at all. Stronger stimulation resulted in frank goiter development without further important changes in the T/S. It is concluded that: (a) hypothalamic damage is not necessary for goitrogen treatment to produce an elevated TS ratio unaccompanied by thyroid enlargement; (b) the capacity of the thyroid gland to trap iodide is a function of its size as well as of its avidity for this ion; (c) the TS ratio is a useful index of thyrotrophic stimulation, but only over a limited range; and (d) a combination of physiological and anatomical parameters of thyroid ac-

tivity is more useful than any one single criterion in assessing the degree of thyrotrophic stimulation.

Acknowledgments

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THE INFLUENCE OF VITAMIN D ON PARATHYROID ACTIVITY AND THE METABOLISM OF CALCIUM AND CITRATE DURING CALCIUM DEPRIVATION[1,2]

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EXPERIMENTALLY (1, 2) and clinically (3) the orthodox action of vitamin D in the absence of the parathyroids is characterized by effects similar to those of parathyroid hormone administration. Harrison and Harrison (4) have shown, however, that in rachitic dogs, vitamin D administration, in contrast to the effect of giving parathyroid extract, results in lessening phosphaturia. More recently, Pincus and Gittleman (5) have presented data indicating that even small doses of vitamin D included in the formulae of newborn infants, especially when these are of high phosphate content, result in lowering of serum calcium and elevation of serum inorganic phosphate concentrations.

These latter, paradoxical effects of the vitamin could indicate that under certain circumstances, particularly situations of physiologically increased parathyroid activity, the vitamin has a direct inhibitory action on parathyroid hormone release. The studies here reported were undertaken to investigate this possibility using rats fed a phosphate rich, nearly calcium free ration to promote intense parathyroid secretory activity. It was reasoned that under these circumstances any tendency for vitamin D directly to suppress the parathyroid response to hypocalcemia or hyperphosphatemia would be readily detectable. It was further reasoned, a priori, that comparison of the behavior of the vitamin D-treated intact rat with that of the parathyroidectomized animal would serve to quantitate the vitamin's inhibitory action if such could be demonstrated.

MATERIAL AND METHODS

Studies were conducted on groups of Sprague-Dawley strain white rats weighing 50 ± 5 gm. at the start of periods of observation. The animals were placed in individual

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wire-bottomed cages, protected from exposure to sunlight, and fed a basic vitamin D-free diet which contained 0.25 mM of calcium and 1.0 M of phosphate per Kg.[3] The amount of diet placed in the food cup of each animal was varied so that essentially the entire amount was consumed each day.

The animals were divided into four groups as indicated in Table 1. Group I animals served as normal controls. They were fed the basic diet supplemented with vitamin D_2 to give an intake of 100 I.U. per day and with calcium carbonate, 125 mM. per Kg. of diet to provide a daily calcium intake of about 1 mEq. per rat. Group II animals were fed the basic diet with no vitamin D or calcium supplement. Group III animals received the same diet with a vitamin D supplement of 10,000 I.U. per day delivered in 0.1 ml. of ethanol. Group IV animals had been parathyroidectomized prior to the period of study. The operation was done on healthy young animals which had attained weights of 40 gm. They were started on the basic low calcium diet supplemented with 10,000 I.U. of vitamin D per day 3 to 5 days postoperatively when their weights had reached approximately 50 gm.

The general health and weight gain of the individual animals were observed daily. At the end of 16 to 23 days observation, the animals were rapidly anaesthetized with 8 mg. per 100 gm. of body weight of sodium nembutal delivered intraperitoneally in a volume of approximately 0.15 ml. Blood was obtained from the abdominal aorta for chemical analysis. After abdominal evisceration x-rays were obtained for visualization of gross changes in bone structure. Thereafter, the remainder of the carcass of representative animals was digested in concentrated nitric acid for total calcium determination. From other representative animals various organs and tissues were removed for chemical analysis and histologic study.

Roentgenograms were taken with the animals in the supine position with extremities extended and held in contact with the film by means of Scotch tape or straight pins. The technical factors used for all films regardless of minor variations in the size of the animals were constant.[4]

Calcium analyses of diaphyseal and epiphyseal bone were done on tibiae containing marrow but dissected free of external soft tissue. The shaft specimens were 5 mm. long sections of the midportion of the bone; the epiphysis specimens consisted of pieces of bone taken from the proximal end of the tibia and included all bone and cartilaginous tissue proximal to a point about 1 mm. distal to the proximal epiphyseal line. The bone specimens were dried to constant weight at 100° C, then ashed and the residue taken up in 0.5N HCl for calcium analysis.

Sodium analyses were done by a micromodification of the method of Wallace (6) using a Barkley flame photometer. Potassium was determined with a Beckman DU flame photometer using a standards containing equivalent sodium. Calcium was precipitated as oxalate, taken up in hydrochloric acid and determined on the Beckman

[3] The composition of this diet was as follows: Na_2HPO_4, 404 gm.; NaCl, 47 gm.; $MgSO_4 \cdot 7\ H_2O$, 100 gm.; NaI, 0.3 gm.; $CuSO_4 \cdot 5\ H_2O$, 0.4 gm.; $ZnCl_2$, 0.09 gm.; $MnSO_4 \cdot 4\ H_2O$, 3.2 gm.; $FeC_6H_5O_7 \cdot 3\ H_2O$, 30 gm.; KCl, 143 gm.; $NaHCO_3$, 182 gm.; cystine, 20 gm.; thiamine HCl, 0.04 gm.; riboflavin, 0.12 gm.; pyridoxin, 0.04 gm.; Na pantothenate, 0.25 gm.; menadione, 0.50 gm.; nicotinic acid, 0.50 gm.; inositol, 10 gm.; p-aminobenzoic acid, 20 gm.; casein, 625 gm.; dextrose, 1,900 gm.; corn oil, 125 gm.; tocopherol, 0.3 gm.; vitamin A, 50,000 units; choline dihydrogen citrate, 20 gm.

[4] The technical factors were as follows: Thirty K.V., 120 ma., 0.75 sec., 36 in F.S.D. and a conventional lead back cardboard holder containing Kodak non-screen film. The same machine was used for all exposures. The films were processed in a standard X-ray developer for 8 min., and fixer for 25 minutes at 68° F and washed for one hour before drying.

flame photometer at 554 mu. Inorganic phosphate was determined according to the method of Fiske and Subbarow (7); alkaline phosphatase by the procedure of Bodansky (8); total protein by a micromodification of the method of Gornall (9) and CO_2 by the method of Van Slyke (10). Serum pH was determined by Hasting's colorimetric method (11). Citrate analyses were done by the procedure of Natelson, Pincus and Lugovoy (12) as modified by Elliott (13). Serum ionized calcium concentration was determined by the McLean and Hastings bioassay method (14) using hearts from Rana pipiens to permit reduction in sample volume to 0.5 ml. or less. Values were also estimated from the nomograms prepared by the same authors (15, 16). Magnesium determinations were performed by the method of Garner (17).

In evaluating the data, mean values obtained from animals within a given group have been compared with those for animals of another group and designated significantly different only when the probability value using the "t" test was equal to or less than 0.05 (18).

RESULTS

The observations made on animals of the various groups are summarized in Tables 1 and 2. The data of Table 1 are representative of the status of non-tetanic animals on the 18th day of their respective special regimens. The data of Table 2 were obtained on the 21st day when all of the Group III animals had begun to manifest tetany.

TABLE 1

	Group I	Group II	Group III	Group IV
Animal Status:	Intact	Intact	Intact	Parathyroid-ectomized
Vit. D; I.U./day:	100	0	10,000	10,000
Calcium; mEq./Kg. diet:	125	0.5	0.5	0.5
Clinical observations (18 days):				
Mean weight gain (gm./day)	5.8	2.5	5.1	2.8
Tetany: (21 days)	0	0	+++	+++
Parathyroid Size:	N	++	N	Absent
Serum conc. values (18 days):				
Calcium: mgm.%	9.8	4.9	8.5	4.6
Inorg. Phos.: mgm.%	8.7	9.6	9.2	15.5
Alk. Pptase.: B.U.%	67	98	58	60
T. P., gm.%	6.2	6.2	5.6	4.9
Citrate, mgm.%	4.5	3.2	5.6	4.0
pH	7.39	7.31	7.41	7.40
CO_2, mEq./L.	26.7	21.9	24.9	30.0
Na., mEq./L.	143	149	144	149
Cl, mEq./L.	99	107	109	106
K, mEq./L.	5.6	4.3	4.5	4.7
Mg., mEq./L.	1.9	2.3	2.2	2.0
Carcass calcium: {mM/animal, evisc.} {At start: 8.55}	–	7.94	7.93	7.18
Calcium balance (per cent change): $100\ X\left(1-\frac{\text{Final carcass calcium}}{\text{Initial carcass calcium}}\right)$:	–	−7.1	−7.2	−8.9
Epiphysis/Diaphysis calcium: {mM/gm. dry weight} {At start 2.69/4.27}	–	1.35/4.55	1.40/2.55	1.67/3.66
Carcass magnesium: (mM/animal, evisc.)	1.52	0.97	1.18	–

The weight gain of the intact, control animals of Group I was normal (Table 1). It was superior to that of animals in the remaining groups and was essentially a rectilinear arithmetic function of time. Weight gain of the animals of the other groups was comparable to that of the controls at the start of the special diets but tended to diminish and in some cases to cease in the latter days of observation. The poorest weight gain throughout was shown by the animals of Group II. Here there was essentially no gain recorded after the first 12 days on the diet. Weight gain of the Group III animals was comparable to that of the controls for the first 15 days on the diet but slowed thereafter. The parathyroidectomized animals of Group IV gained weight more slowly than the similarly fed intact animals of

TABLE 2. INFLUENCE OF VITAMIN D ON CALCIUM AND CITRATE METABOLISM
(Data based on observations at 21 days)

Group II (No vitamin D)	Group III (Vitamin D, 10,000 I.U./day)
Serum	
Total calcium (mgm. per cent): 5.3	8.3
Total protein (gm. per cent): 4.8	4.8
Citrate (mgm. per cent): 3.0	5.5
Ionized calcium	
Frog heart (mgm. per cent): 2.2	3.6
Nomogram (mgm. per cent): 2.4	3.2
Tissue citrate (micrograms per gm. wet tissue):	
Brain: 32	29
Muscle:	
Abd. wall: 23	36
Thigh: 25	38
Kidney: 86	326
Skeleton: 910	1850

Group III. Their weight curve also showed a tendency to flatten after the 15th day of special diet.

Tetany was absent in the animals of Group I. It was similarly absent in the Group II animals at 18 days but mild tetanic tremors began to appear at 23 days on the vitamin D-free, low calcium ration. No haemorrhagic tendencies were noted even after 4 weeks on the diet. By contrast, severe tetany which resulted in death 12 to 24 hours after the development of the first symptoms was observed in both the Group III and Group IV animals. This tetany appeared suddenly and with considerable regularity between the 18th and 21st days of observation. The tetanic symptoms of both the vitamin D-deficient (Group II) and high vitamin D-fed animals (Groups III and IV) could be alleviated temporarily by respiratory acidosis produced by rebreathing. In the vitamin D-fed animals, onset of tetany could be delayed several days by supplementing the individual food ration with 0.5 mM. of calcium as calcium carbonate once on the 17th day. Tetany was also alleviated for a few hours' time by intravenous administration of 0.012 mM. calcium as calcium gluconate. Severe tetany could be

provoked within 3 days in the Group II animals by introducing into the diet 10,000 I.U. daily of vitamin D after 20 days on the vitamin D-free ration.

After sacrifice the parathyroids were exposed and examined under a binocular dissecting microscope at 10× magnification in a total of 15 animals containing equal numbers of each of the three groups of intact rats. To avoid bias, one of us (J.D.C.), who had no knowledge of the diet group to which the individual animals belonged, examined the parathyroids in situ. The observer was unable to make a distinction on the basis of size or vascularity between glands of the Group I and Group III animals. The glands of the Group II animals were readily distinguished from those of the other two groups on the basis of their larger size and increased blood supply. Only one error was made by the observer in the proper assignment of animals to this group.

In handling the animals during the dissection of the parathyroids and other tissues for chemical and histologic study, it was noted that the bones of all save those of Group I were soft. Marked craniotabes was present. x-ray studies, described in more detail below, showed multiple fractures to be present in the long bones. The absence of callus or soft tissue swelling about the fracture sites indicated that they were the result of post mortem manipulation.

The blood chemical analysis values shown in Table 1 represent means of determinations made on samples from representative animals in the various groups. With the exception of the pH and CO_2 values for animals in Group IV, each of which represent averages of three determinations only, the means are based on at least six analyses. Variations about the means were minor.

The serum concentration values recorded for the Group I animals can be considered normal throughout.

The total serum calcium concentration in the Group II animals was found markedly reduced. In the Group III animals, the total serum calcium concentration of 8.5 mg. per 100 ml. was much higher than in the rats of Group II. This mean value, however, is significantly lower than that found in the Group I controls. The total serum calcium concentration in the parathyroidectomized animals of Group IV was depressed to an extent approximately equal to that found in the intact, D deficient animals of Group II.

Mean serum inorganic phosphorus concentration values of the animals in Group II and III were slightly higher but not significantly different from those of the Group I controls. In the animals of Group IV, serum inorganic phosphorus concentrations were markedly and significantly elevated.

Serum alkaline phosphatase concentrations in the control animals of Group I gave a mean value of 67 Bodansky units per cent. In the much more slowly growing members of Group II, the mean value of 98 Bodansky units per cent is significantly higher than the control value or the values found in animals of Groups III and IV. The mean values of the animals in these two latter groups, 58 and 60 Bodansky units per cent respectively,

are not significantly different one from the other but both means are significantly below the value for the Group I controls.

Serum total protein values were normal in the Group II rats; a moderate reduction was found in animals of Group III and even lower values were determined in the members of Group IV.

The mean serum citrate concentration of the control animals, (Group I) was 4.5 mg. per 100 ml. The mean value obtained in the animals of Group II, 3.2 mg.%, was significantly reduced relative to the control value. In the animals of Group III the mean of 5.6 mg.% is significantly above that of the Group I controls as well as that for the animals in Group II. In the animals of Group IV the mean serum citrate concentration of 4.0 mg.% lies approximately midway between the values found in Group I and II animals and is significantly below the mean for animals of Group III.

Hydrogen ion and CO_2 values obtained in the Group II animals are indicative of slight acidosis while those of the remaining two groups are approximately normal. The mean values for serum sodium concentration show no significant differences between the various groups. The mean serum chloride concentration of the control animals is lower than those obtained in Groups II, III and IV. Serum potassium concentration was found slightly higher in the Group I animals than in the remaining study groups. Serum magnesium concentrations were in the normal range in all groups.

Total carcass calcium after abdominal evisceration was determined in three normal 50 gm. animals comparable to those which were assigned to the intact study groups. The average of these analyses, 8.55 mM. per animal, can be compared with the analyses performed on three carcasses from each group at the end of the special feeding periods.

These analyses showed a mean calcium content of 7.94 mM. per animal for the Group II rats and 7.93 mM for the rats of Group III. The results indicate that approximately equal losses of body calcium had occurred in these two groups of intact animals during the 18 days of low calcium feedings. Final carcass calcium analyses of five animals of Group IV yielded a mean value of 7.18 mM. per animal. For estimation of calcium balance this mean must be compared with a prestudy figure for total body calcium of comparable parathyroidectomized rats (7.87 mM/animal). Such a comparison indicates that the animals of Group IV lost about 9% of the total body calcium during the study period, a loss probably not significantly different from the losses of the animals of Groups II and III.

Analyses of the calcium content of epiphyseal and diaphyseal bone were made on specimens from normal, 50 gm. animals comparable with those assigned the various study groups. These permit comparison with the values found in the specimens from the animals of groups II, III and IV. Marked demineralization of epiphyseal bone was evident in all specimens from these groups. Diaphyseal bone showed a calcium content comparable

with that of starting controls in the animals of Group II. The calcium content of diaphyseal bone of Group III was markedly depressed while that of the Group IV animals was intermediate between the very low value for the Group III animals and the "normal" values for the animals of Group II.

Table 2 gives further data with respect to serum calcium concentrations and the concentration of citrate in serum, tissue and bone. Determinations were made only for intact animals fed the low calcium diet for 21 days either without vitamin D (Group II) or supplemented with 10,000 I.U. daily (Group III). The mean serum total calcium and citrate values are similar to those of comparable groups of Table 1. Total protein concentrations in these animals were significantly lower probably due to the longer period of observation prior to sacrifice and the fluid retention which accompanies impending or manifest tetany (19). There was, however, no influence on serum protein attributable to vitamin D. Serum ionized calcium concentration was estimated from the McLean-Hastings nomograms as well as determined by their frog heart method (14–16). The latter determination was done in order to investigate the possibility that some polycarboxylic acid or other unmeasured substance in serum was acting to depress the ionized calcium concentration of the tetanic Group III animals below that of the non-tetanic animals of Group II. As can be seen in the Table, agreement between the nomographic estimation and frog heart determination was good. Representative kymographic tracings are shown in Figure 1.

Tissue citrate concentrations are shown in the lower portion of Table 2. Here it is evident that in all tissues examined with the exception of brain, there was an elevation of citrate concentration in association with the giving of large doses of vitamin D. The elevation was particularly marked in bone and kidney. In the case of kidney tissue the accumulation of citrate was even more marked than is evident from the concentration values since the mean kidney weight of the vitamin D-treated rats (2.18 gm.) was nearly double that of the vitamin D deficient animals of Group II (1.16 gm.).

The x-ray appearance of the bones of animals in various study groups is reproduced in Figure 2. The original films were examined by the authors as well as by two observers[5] who had a general idea of the problem presented but did not know to which group of animals the various films belonged. All agreed that the findings enumerated below were characteristic.

The density of the bones of the animals of Group II, III and IV (Fig. 2-B, C and D) was significantly less than that of the control animals of

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[5] We are indebted to Dr. Laurence L. Robbins and Dr. Joseph Hanelin for their help in interpreting these films.

Group I (Fig. 2-A). In the Group II animals the skeletons were smaller than in any other group. There was widening of the epiphyses and flaring of the metaphyses evident at many joints. The bones were small and deformed but the trabecular pattern was fairly well preserved. The changes were interpreted as being typical of rickets.

In the animals of Group III (Fig. 2-C), the skeletons were almost as large as those of the Group I controls. Bowing of the long bones and demineralization, especially of the diaphyseal bone, were more marked than in the animals of Group II. In addition there was greater loss of detail of bone structure and trabeculae in the animals of Group III as compared

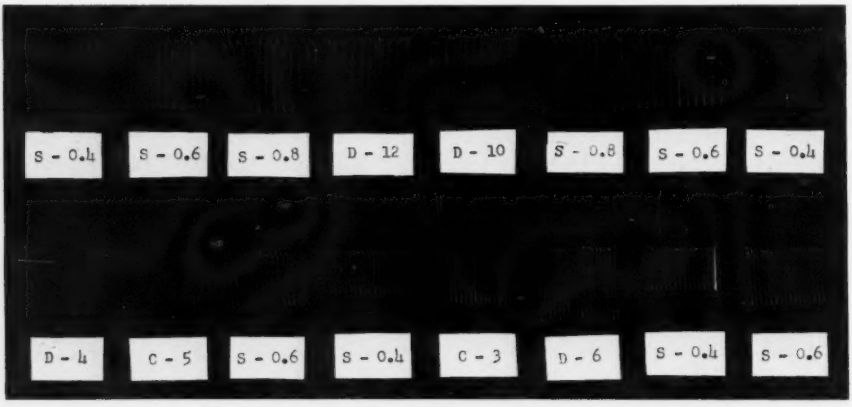

FIG. 1. Sequential kymographic tracings of frog heart response to perfusion with rat serum and buffered calcium standards. Values following the letter S refer to the millimolar calcium concentration of buffered standards. Values following the letter D refer to the individual classification number of rats treated with 10,000 I.U. of vitamin D_2 daily (Group III); those following the letter C to the classification number of rats on diet lacking vitamin D (Group II).

with those of Group II. Widened epiphyses and flared metaphyses were not present in Group III.

The x-ray appearance of the bones of the animals of Group IV (Fig. 2-D) was transitional between that of the Group II and III animals in terms of size, density and loss of detail. As in the Group III animals there was no widening of the epiphyses nor flaring of the metaphyses. The findings in this group as well as in the animals of Group III were interpreted as consistent with osteoporosis.

Histologic examinations were made of bone, kidneys and parathyroids removed from representative animals of the various groups. No abnormalities were found in the kidneys though those of the Group III animals

were distinctly larger than those of the controls. Except for the hyperplasia in the animals which received no vitamin D, the parathyroid glands showed no morphologic change. Representative bone sections are shown in Figure 3. The striking feature of those of the Group II animals which received no vitamin D or calcium in the diet is the large amount of osteoid surrounding the bone trabeculae both in the cortical bone and in the zone of provisional calcification below the epiphyseal cartilage (Fig. 3-B). This is in sharp contrast to the control animal which shows immature bone but no osteoid (Fig. 3-A). In the section from the intact Group III animals which received the large doses of vitamin D (Fig. 3-C) there is no demonstrable osteoid but the bone trabeculae are more deeply stained suggesting increased deposition of calcium. In the parathyroidectomized animals which received large doses of vitamin D, many of the bone trabeculae are surrounded by small but definite amounts of osteoid (Fig. 3-D).

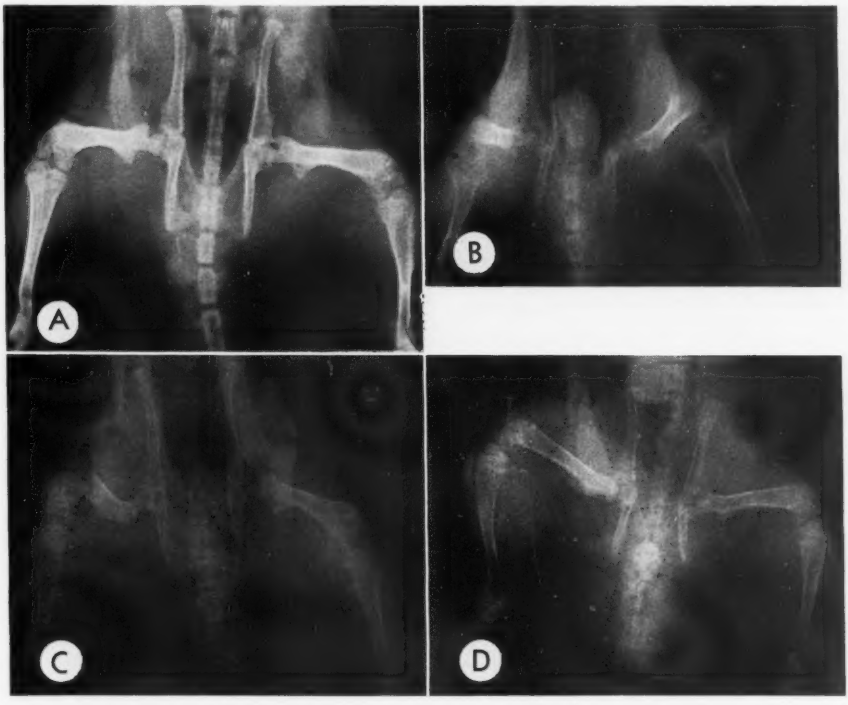

FIG. 2. X-Rays of representative animals from each of the study groups. A. Normal control (Group I); B. intact, calcium and vitamin D-deficient (Group II); C. intact, calcium deficient animal given 10,000 I.U. of vitamin D daily for 18 days (Group III); D. parathyroidectomized, calcium deficient animal given 10,000 I.U. of vitamin D daily for 18 days (Group IV).

DISCUSSION

The foregoing evidence suggests that Vitamin D in the doses here employed has no direct parathyroid secretory suppressive action. Though there was relatively early development of apparent tetany in the intact animals treated with large doses of the vitamin (Group III) and the parathyroids in these animals were grossly normal in contrast to the enlarged, hyperemic glands of the vitamin deficient group (Group II), other explanations for these phenomena appear to be in order. Serum analyses for total calcium showed distinctly higher levels in the tetanic, vitamin D-treated

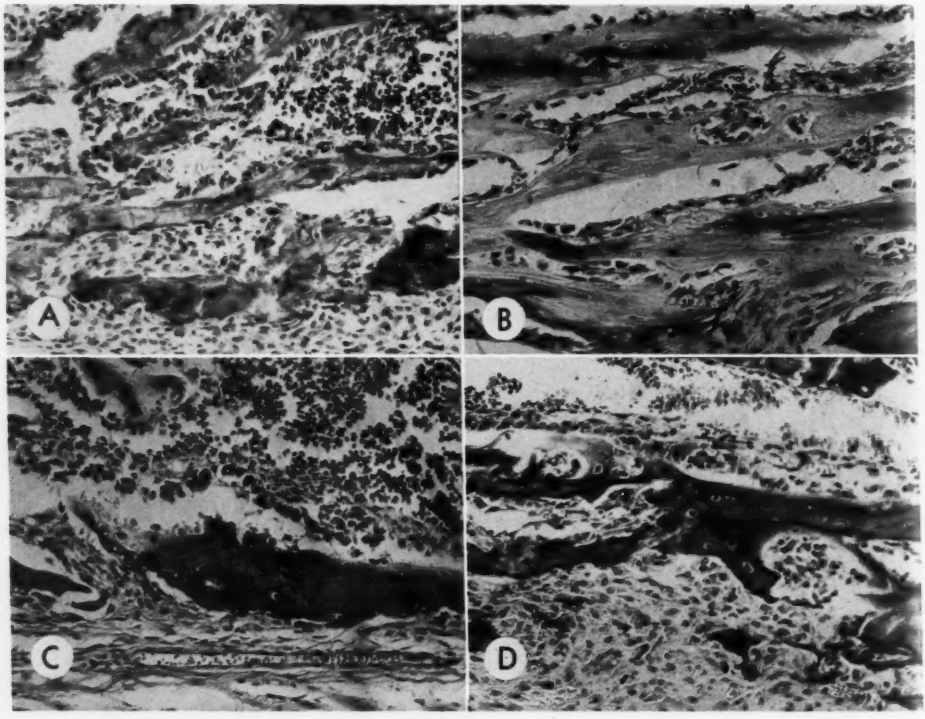

FIG. 3. Photomicrographs of cortical bone from representative animals of each of the study groups (×200). A. Normal control (Group I); B. intact, calcium and vitamin D deficient (Group II); C. intact, calcium deficient given 10,000 I.U. of vitamin D for 18 days (Group III); D.parathyroidectomized, calcium deficient animal given 10,000 I.U. of vitamin D for 18 days (Group IV).

animals of Group III. Though vitamin D caused as expected, an elevation of serum citrate concentration, the magnitude of the hypercitricemia was insufficient to cause a major reduction in the ionized moiety of total serum calcium. As is evident in Table 2, serum ionized calcium calculated from total calcium, protein and citrate values was higher in the non tetanic,

Group III animals than in those of Group II. Table 2 and the tracings of Fig. 1 further indicate that biologic determination of serum ionized calcium concentration failed to yield evidence in the vitamin D treated rats of circulating constituents other than protein and citrate with appreciable calcium binding properties. The data relative to serum concentration values thus fail to yield an explanation for the "tetany" of the intact, vitamin D-treated rats though the near normalcy of the calcium concentration values does appear to provide an explanation for the lack of parathyroid hypertrophy noted in these rats.

Inasmuch as serum analyses failed to provide an explanation for the tetany, an exploration of tissue citrate concentrations was undertaken. Steenbock and Bellin have shown that vitamin D augments tissue citrate content (20). Buffa and Peters (21) have used fluoracetate to poison aconitase, the enzyme responsible for the equilibrium between the C-6 acids of the citric series, citrate, cis-aconitate and isocitrate (22). Their studies have shown that normocalcemic tetany can be produced in rats as a result of tissue citrate accumulation, presumably that in nervous tissue being chiefly responsible. The pertinent data of the present study shown in the lower section of Table 2 indicate that under the influence of large doses of vitamin D, citrate becomes moderately elevated in muscle and markedly increased in kidney and bone. In contrast to the influence of fluoracetate or fluorcitrate, vitamin D fails to cause excess citrate accumulation in brain tissue. Thus, unless the 50% increase in muscle citrate observed in the vitamin D treated animals accounts for the tetany or there were citrate accumulations in peripheral nervous tissue not reflected in the analyses of brain, the chemical data must be judged inadequate to provide an explanation for the convulsive disorder observed.

Comparison of the data for vitamin D-treated intact (Group III) and parathyroidectomized animals (Group IV) provides clear indication that the parathyroids, when present, continue to play an active secretory role during calcium deprivation and treatment with these doses of vitamin D. Perhaps the most striking manifestations of this are the lower serum calcium and much higher serum inorganic phosphorus concentrations in the operated animals. Indeed, comparison of the biochemical data for the intact, vitamin D-treated animals of Group III with those both for intact animals not receiving vitamin D (Group II) and parathyroidectomized animals treated with the vitamin (Group IV), suggests not only continued production of parathyroid hormone during vitamin D treatment but also similarity of action and synergism between the two agents.

It has been convincingly shown that parathyroid hormone has a direct demineralizing action on bone (23, 24). Prior evidence (25, 26) as well as the present data suggest that vitamin D, at least in heavy doses, has a similar action. Mobilization of skeletal calcium stores by parathyroid hormone in the animals of Group II, by both agents in Group III and by vita-

min D alone in Group IV would seem to be of prime importance to their relatively long survival. Rats lacking both parathyroid hormone and vitamin D die of hypocalcemic, hyperphosphatemic tetany in less than 72 hours on the diet used. It would seem indicated by the data of Table 1 relative to the calcium content of epiphyseal and diaphyseal bone as well as by the x-rays of Figure 2 that the mobilization of calcium to maintain plasma homeostasis due to parathyroid hormone occurs primarily in the epiphyseal area with sparing of diaphyseal deposits. Vitamin D, on the other hand, appears to facilitate such mobilization of calcium from both sites. In a small series of rachitic animals of Group II treated for three days with vitamin D after 20 days on the low calcium diet, serum calcium concentrations became elevated to levels nearly as high as those of the Group III animals. It seems likely that the calcium necessary for this came from the relatively rich diaphyseal bone stores though considering the very small amounts of calcium required, diet or other sources cannot be excluded.

SUMMARY AND CONCLUSIONS

Intact, 50 gm. rats fed a calcium free diet supplemented with 10,000 I.U. of vitamin D daily develop a severe convulsive disorder suggestive of tetany after approximately 20 days' feeding. Much milder tetany of later onset is observed in calcium deprived, vitamin D deficient animals. In the vitamin treated animals no parathyroid hypertrophy is observed, chemical studies show near normal serum values for total and ionized calcium and tissue analyses indicate citrate accumulation in muscle, bone and kidney but not in brain. There is diffuse, osteoporotic decalcification of bone. Intact rats fed the same diet without vitamin D show parathyroid hypertrophy, lower total and ionized serum calcium concentrations and rachitic bone lesions with decalcification most marked at the epiphyses. Comparison of the behavior of intact and parathyroidectomized animals fed the low calcium, high vitamin D diet indicates that parathyroid secretory activity continues in intact animals fed these large vitamin D intakes and that the vitamin and hormone in this circumstance have synergistic actions.

The present experiments fail to yield an explanation for the vitamin's so called paradoxical action in rickets or in the human newborn fed cow's milk. It is suggested that with heavy doses of vitamin D normocalcemic tetany may result from citrate accumulations in or about peripheral nervous tissue.

Acknowledgment

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METABOLISM OF ADIPOSE TISSUE *IN VITRO*: NUTRITIONAL FACTORS AND EFFECT OF INSULIN

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THE oxygen consumption of adipose tissue was investigated by Mirski (1). More recently Haugaard and Marsh (2) found that addition of insulin *in vitro* increases the oxygen consumption of retroperitoneal body fat in the presence of glucose, lactate, succinate, pyruvate or acetate. Although the utilization of glucose by muscle (rat diaphragm) and the stimulating effect of insulin on this process have been extensively studied (reviews 3, 4), very little is known concerning carbohydrate metabolism of adipose tissue. Krahl (3) reported that rat adipose tissue utilizes glucose *in vitro* and that this utilization is also stimulated by insulin. Furthermore, insulin enhances glycogen deposition in adipose tissue *in vivo* (5, 6, 7, 8). Fawcet (9) concluded that insulin *in vivo* has a direct local action on the adipose tissue at the site of injection.

The nutritional state of the animal seems to play an important role in regulating the metabolic activities of adipose tissue. Glycogen deposition in adipose tissue is known to be influenced by the diet (10). Thus the incorporation of deuterium into fatty acids of adipose tissue *in vitro* is greater in tissues excised from rats refed on a carbohydrate-rich diet after a period of food restriction, than in tissue from normally fed animals (1).

In the light of these studies it was considered of interest to study glucose utilization and oxygen consumption by the adipose tissue of rats in different nutritional states *in vitro*, and the effect of insulin on these processes.

METHODS

Animals: Male albino rats of an average weight of 100–130 gm. (about 3 months old), from a local strain which attains a mature weight of about 250 gm. in 6 to 7 months were used.

Starvation Regime: For starvation experiments the rats were fasted for 1 to 5 days. During this period water was given *ad lib.* Two days' fast resulted in about 15% and 5 days' fast in about 30% loss in body weight. For the refeeding experiments, unless otherwise specified, the rats were fasted for 5 days and then refed on a stock diet or carbohydrate-rich diet for 1–14 days.

Diets: The stock diet was a mixture of soya bean meal, dried beef, wheat bran, oil cakes and wheat.

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The composition of the experimental diets was as follows:
(a) *Carbohydrate-rich diet:* 67% starch, 18% casein, 9% fat, 3% dried yeast and 3% salt mixture.
(b) *Protein-rich diet:* 18% starch, 67% casein, 9% fat, 3% dried yeast and 3% salt mixture.
(c) *Fat-rich diet:* 12% starch, 18% casein, 64% fat, 3% dried yeast and 3% salt mixture.

Preparation of the tissues: The rats were anaesthetized with pentobarbital and bled. Mesentery was stripped off the intestine, dissected free of pancreatic tissue and of lymph nodes, and cut into a few pieces. Testicular adipose tissue was torn from the epididymis. The interscapular brown adipose tissue was removed and dissected free of the surrounding white fat and muscle. The thick parts of the testicular and of the interscapular fat were sliced with a Stadie-Riggs microtome (12) while the thinner parts were used without further treatment. Before incubation all tissues and slices were rinsed in ice-cold saline containing 0.688% NaCl, 0.018% KCl and 0.027% $CaCl_2$.

Incubation medium: Krebs-Ringer phosphate solution (pH 7.4) was used. It contained 0.9% NaCl, 100 parts; 1.15% KCl, 4 parts; 1.22% $CaCl_2$, 3 parts; 3.8% $MgSO_4 \cdot 7H_2O$, 1 part; a mixture of 40 ml. of 0.25 M Na_2HPO_4 and 2 ml. of N HCl brought to a volume of 100 ml. and to pH 7.4, 21 parts; glucose content, 0.1%.

Measurements of respiration and glucose utilization: Determination of oxygen consumption was carried out by the direct method of Warburg (13) using 1.5 ml. of medium and 0.2 ml. of 10% KOH in the center cup. The flasks were gassed with oxygen for 5 minutes, placed in a water bath at 37° and shaken at the rate of 110 excursions/min. for 2.5 hr. Fifteen minutes were allowed for equilibration. Readings were made every 30 minutes. At the end of the experiment glucose in the medium was determined by the method of Somogyi (14). The rate of glucose utilization by the tissue was calculated from the difference between the initial and the final glucose concentrations. Tissues from 2 rats were used for each of the experiments carried out with mesentery, and tissues from 3 rats were taken for the experiments with testicular and brown fat. For the experiments with insulin, 2 Warburg flasks containing equal parts of tissue from each animal were used, one flask with and one without insulin. The insulin (Squibb) was added to the medium before each experiment to give a final concentration of 1 unit per ml.

Determination of fat-free dry weight of tissue: At the end of the incubation period, the tissue from each Warburg flask was transferred to an Erlenmeyer containing 10 ml. of alcohol-acetone (1:1) and left overnight. The liquid was then decanted and 10 ml. of ether were added. Extraction of fat was carried out by bringing the ether to the boiling point, leaving the flask for about 1 hr. at room temperature, and bringing the ether to boil again. The tissue was then taken out and pressed between pads of filter paper. The extraction procedure was repeated using a fresh portion of ether. The dry, defatted tissue was placed in an oven at about 50° C for 20 min. and then weighed. Per cent dry defatted tissue was calculated from the ratio:

$$\frac{\text{wt. of dry, defatted tissue}}{\text{wt. of fresh tissue}} \times 100.$$

Computations of results: Glucose utilization was expressed as mg. glucose/10 mg. dry, defatted tissue/2.5 hr. The rate of oxygen consumption was expressed as /μl. O_2/mg. dry defatted tissue/hr. during the first 2 hours of respiration. Estimations on such a basis have several advantages over the usual practice of calculating the results per unit weight of wet tissue (3, 2, 17). They permit the evaluation of the actual metabolic activities of a given tissue on a standard reproducible basis, since these activities are usually correlated with the protein content of the tissue. They also enable us to compare

rates of biochemical processes occurring in different tissues with those taking place in adipose tissue, where under different conditions the enzymatically-active contents of the cell are diluted to various degrees by inert matter, such as fat and water. Furthermore, this is technically simpler and less time-consuming than calculation of the results on the basis of the nitrogen content of the tissue.

Using the present method of calculation, it was noted that glucose utilization by white adipose tissue *in vitro* (about 0.400 mg./10 mg. dry defatted tissue/2.5 hr.) is of the same order of magnitude as glucose utilization by muscle. The average value for glucose utilization by the isolated diaphragm obtained in this laboratory under similar experimental conditions was 0.600 mg./100 mg. wet tissue/2.5 hr., equivalent to 0.330 mg./10 mg. dry defatted tissue/2.5 hr. However, the rate of oxygen consumption by white adipose tissue was much lower than that calculated for the diaphragm (about 2/μl. O_2 as compared with 10/μl. O_2/mg. dry defatted tissue/hr.).

RESULTS

Mesenteric fat: Glucose utilization decreased at first on fasting but after 4 days the control level was again reached. Oxygen consumption increased progressively during the first 4 days of fasting (Table 1). Refeeding after fasting for 5 days had no influence on glucose utilization. However, oxygen consumption continued to rise during the first 2 days of refeeding, reaching a level 80% higher than the control value. Thereafter the oxygen consumption began to decrease and reached the control level by the fourteenth

TABLE 1. GLUCOSE UTILIZATION AND OXYGEN CONSUMPTION BY MESENTERIC ADIPOSE TISSUE OF FED, FASTED AND REFED DATS

The tissues were incubated at 37° for 2.5 hr. in Krebs-Ringer phosphate solution containg 0.1% glucose. Results are given as mean ±S.E.M.

Procedure	No. of expts.	Glucose utilization (mg./10 mg. dry defatted tissue/2.5 hr.	P	Oxygen consumption (μl./mg. dry defatted tissue/hr.)	P	Dry defatted tissue (% of wet wt.)
A. *Fasting*						
Normal, fed	27	0.404 ±0.028		1.81 ±0.19		3.33
Fasted 1 day	11	0.379 ±0.025		2.04 ±0.22		3.66
Fasted 2 days	6	0.282 ±0.020	<0.01*	2.07 ±0.32		4.00
Fasted 3 days	6	0.384 ±0.051		2.39 ±0.38		6.12
Fasted 4 days	7	0.424 ±0.031		2.72 ±0.17	<0.01*	7.81
Fasted 5 days	13	0.421 ±0.023		2.28 ±0.17		8.52
B. *Refeeding with stock diet after 5-day fast*						
Refed 1 day	7	0.440 ±0.037		2.92 ±0.39		8.09
Refed 2 days	7	0.413 ±0.079		4.12 ±0.63	0.01**	7.93
Refed 4 days	5	0.316 ±0.064		3.66 ±0.42	0.01**	5.68
Refed 7 days	8	0.384 ±0.070		3.18 ±0.94		4.47
Refed 14 days	3	0.407		2.65		3.53
C. *Refeeding with a carbohydrate-rich diet after 5-day fast*						
Refed 1 day	6	0.335 ±0.030	0.04	3.32 ±0.36	0.02	6.24
Refed 2 days	7	0.586 ±0.061	0.02	5.04 ±0.57	<0.01	7.31
Refed 4 days	4	0.389 ±0.088	0.80	3.85 ±0.62	0.03	5.90
Refed 7 days	8	0.726 ±0.047	<0.01	4.50 ±0.34	<0.01	4.52
Refed 14 days	5	0.481 ±0.055	0.30	2.45 ±0.12	0.50	3.91

* Significantly different from the mean value of normal, fed rats.
** Significantly different from the mean value of rats fasted for 5 days.

TABLE 2. EFFECT OF INSULIN ON GLUCOSE UTILIZATION BY MESENTERIC ADIPOSE TISSUE OF FED, FASTED AND REFED RATS

The tissues were incubated at 37° for 2.5 hr. in Krebs-Ringer phosphate solution containing 0.1% glucose. Tissues from 2 rats were used for each experiment. The effect of insulin is represented by the difference in glucose utilization between two Warburg flasks containing equal parts of tissue from the same animal. Insulin was added to one of the flasks in a final concentration of 1 unit/ml. medium. Results are given as mean ±S.E.M.

Procedure	No. of expts.	Effect of insulin on glucose utilization (Mg./10 mg. dry defatted tissue/2.5 hr.)	Per cent	significance of the effect (P)	P*
A. *Fed, and fasted rats*					
Normal, fed	27	+0.064 ±0.012	+16	<0.01	
Fasted 1 day	11	+0.090 ±0.029	+24	0.01	0.40
Fasted 2 days	6	+0.066 ±0.019	+23	0.02	0.90
Fasted 3 days	6	+0.132 ±0.046	+34	0.04	0.15
Fasted 4 days	7	+0.263 ±0.048	+62	<0.01	0.01
Fasted 5 days	13	+0.134 ±0.024	+32	<0.01	0.01
B. *Rats fasted for 5 days and subsequently refed with stock diet*					P**
Refed 1 day	7	+0.121 ±0.028	+28	<0.01	0.80
Refed 2 days	7	+0.081 ±0.028	+20	0.03	0.20
Refed 4 days	5	+0.226 ±0.034	+72	<0.01	0.05
Refed 7 days	8	+0.233 ±0.042	+61	<0.01	0.05
Refed 14 days	3	+0.147	+36		
C. *Rats fasted for 5 days and subsequently refed with a carbohydrate-rich diet*					P**
Refed 1 day	6	+0.125 ±0.028	+37	<0.01	0.80
Refed 2 days	7	+0.156 ±0.030	+27	<0.01	0.50
Refed 4 days	4	+0.288 ±0.107	+74	0.08	0.20
Refed 7 days	8	+0.698 ±0.070	+96	<0.01	<0.01
Refed 14 days	5	+0.304 ±0.024	+63	<0.01	<0.01

* Statistical comparison with normal, fed rats.
** Statistical comparison with rats fasted for 5 days (part A of the table).

day (Table 1). In none of the groups of animals tested did glucose affect the rate of oxygen consumption.

Insulin increased the glucose utilization of mesenteric tissue of fed rats by 16%. During fasting this effect of insulin was more marked and reached a maximum after 4 days of fasting. Refeeding for 4 to 7 days with our

TABLE 3. GLUCOSE UTILIZATION BY RAT TESTICULAR AND BROWN INTERSCAPULAR ADIPOSE TISSUE

Incubation of tissues and other conditions as in Table 2, except that tissues from 3 instead of 2 rats were used for each experiment.

Procedure	No. of expts.	Effect of insulin on glucose utilization: Glucose utilization (mg./10 mg. dry defatted tissue/2.5 hr.)	Mg./10 mg. dry defatted tissue/2.5 hr.	Per cent	Significance of the effect (P)
A. *Testicular fat*					
Normal, fed	12	0.450 ±0.031	+0.101 ±0.035	+22	0.02
Fasted 5 days	12	0.405 ±0.040	+0.085 ±0.027	+20	0.01
B. *Interscapular fat*					
Fasted 5 days	8	0.184 ±0.007	+0.044 ±0.012	+24	0.01

stock diet after a fast of 5 days resulted in an even more pronounced response to insulin. Thereafter the effect of insulin on glucose utilization decreased until after 14 days of refeeding the control level was reached. When the rats were refed a carbohydrate-rich diet, the insulin effect was still more marked (Table 2). Insulin had no effect on oxygen consumption by mesenteric tissue under any of the nutritional conditions assayed.

Testicular and intrascapular fat: Testicular adipose tissue utilized glucose at the same rate as mesenteric tissue under the conditions studied. However, interscapular adipose tissue used much less glucose (rats fasted for 5 days, Table 3). Although fasting for 5 days did not alter the oxygen consumption of either testicular or brown interscapular fat, refeeding induced a sharp increase in the rate of oxygen consumption (Table 4).

TABLE 4. OXYGEN CONSUMPTION BY TESTICULAR AND BROWN INTERSCAPULAR ADIPOSE TISSUE OF FED, FASTED AND REFED RATS

Refeeding was carried out with stock diet after a 5-day fast. The tissues were incubated at 37° for 2.5 hr. in Krebs-Ringer phosphate solution containing 0.1% glucose. Results are given as mean ±S.E.M.

Procedure	No. of expts.	Oxygen consumption (/μl./mg. dry defatted tissue/ 2.5 hr.)	P*	Dry defatted tissue (% of wet wt.)
A. *Testicular fat*				
Normal, fed	12	2.29 ±0.38		1.36
Fasted 5 days	12	1.51 ±0.17		3.52
Refed 2 days	5	2.99 ±0.56	0.02	4.33
Refed 4 days	7	4.62 ±0.86	<0.01	4.11
B. *Interscapular fat*				
Normal, fed	9	2.70 ±0.64		6.35
Fasted 5 days	8	3.14 ±0.26		7.70
Refed 2 days	8	6.63 ±0.62	<0.01	7.27

* Statistical comparison with rats fasted for 5 days.

Insulin increased glucose utilization by testicular tissue of normal rats. Fasting the animals for 5 days did not influence this insulin effect, in contrast with the results obtained for mesentery (Table 3).

DISCUSSION

The significant fact emerging from the present study is that the antecedent nutritional state of the animal is of prime importance in determining the glucose utilization and oxygen consumption of its adipose tissue and the degree of response of the adipose tissue to insulin *in vitro*. In some groups, the rate of oxygen consumption of adipose tissue was two to three times higher in the refed than in the normal rats. In this connection, it is noteworthy to point out that similar refeeding conditions are known to favour deposition of glycogen (15) and to accelerate fat synthesis in adipose tissue *in vitro* (16). The high rate of oxygen consumption by adipose

tissue of refed rats is a further indication of the active metabolic state of this tissue during recovery feeding. This shows that oxidative processes take place at a higher rate, presumably to meet the extra energy required for the enhanced synthetic processes, such as fat and glycogen synthesis.

Not only does nutritional state influence the metabolism of adipose tissue, but it also alters the sensitivity of adipose tissue to a hormone, insulin. This change in sensitivity may be specific for adipose tissue, since for other tissues, such as muscle, the effect of insulin is constant, despite changes in nutritional state of the type investigated. Our results concerning the effect of insulin on oxygen consumption are out of keeping with those reported by Haugaard and Marsh (2). Working with retroperitoneal fat of rats fasted for 24 hours, these investigators found that insulin increases the oxygen consumption in the presence of glucose. However, Breibart and Engel (17), using a similar technique, could not confirm these results.

It should be mentioned that the changes observed in the rate of glucose utilization, the degree of the stimulating effect of insulin on glucose utilization, and the rate of oxygen consumption could not be correlated with changes in the ratio: fat-free dry wt./wet wt. of adipose tissue. This ratio is an index of the physical state of the animal and of the fat content of its adipose tissues.

Comparison of the types of adipose tissue studied here reveals that brown interscapular fat from fed, fasted and refed rats respires at a higher rate than mesenteric and testicular fat. This finding is constant with the view that brown adipose tissue has higher metabolic activities than white adipose tissue (1, 17, 18). On the other hand, glucose utilization seems to be lower in brown than in white fat, as shown in rats fasted for 5 days.

SUMMARY

The *in vitro* glucose utilization and oxygen consumption by adipose tissue of rats, as well as the effect of insulin on both processes, were studied. The tissues were taken from animals subjected to various nutritional conditions.

Oxygen consumption by mesenteric adipose tissue of rats increased with fasting up to 4 days. It was further increased by refeeding after the fast.

Glucose utilization by mesenteric tissue was not markedly influenced by most of the various nutritional conditions studied.

Insulin increased glucose utilization by mesenteric tissue to a greater extent in fasted rats (4–5 days) than in fed rats. Refeeding especially with a carbohydrate-rich diet, further intensified this effect of insulin. Insulin however had no significant effect on oxygen consumption of adipose tissue in these experiments.

It is concluded that the nutritional state of the animal is important in

determining the metabolic activity and sensitivity to insulin of adipose tissue.

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THE RESPONSE OF ISOLATED RAT DIAPHRAGM TO THYROXINE *IN VITRO*

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ALTHOUGH a number of tissues taken from thyroxine-treated animals have been shown to use oxygen at an elevated rate (1, 2, 3), evidence on the metabolic response of isolated tissues to thyroxine *in vitro* remains inconclusive (1, 3, 4). Since there is a delay of a few hours before a measurable response occurs even when thyroxine is administered *in vivo* (2, 5), it seems probable that isolated tissues would require a similar interval before responding to thyroxine *in vitro*. However, because many investigators concluded their experiments after one or two hours, they may have missed the effect they sought.

In this study, two methods were used in an attempt to obviate thyroxine's latent period: 1) isolated tissues were allowed to remain in contact with thyroxine for as long as 8 hours, and 2) tissues were "primed" by pretreating the donor animals with a single large dose of thyroxine 20 hours prior to removal of the tissue.

MATERIALS AND METHODS

Diaphragms from 94 weanling rats weighing about 50 gm. each were divided into 2 main groups: 1) those obtained from normal, untreated animals, and 2) those obtained from animals receiving 0.3 mg. of DL-thyroxine 20 hours prior to sacrificing. Each of these groups of diaphragms was subdivided into 3 subgroups: 1) control diaphragms, not exposed to thyroxine *in vitro*, 2) diaphragms exposed to 2×10^{-6} *M* thyroxine *in vitro*, and 3) those exposed to 2×10^{-5} *M* thyroxine *in vitro*.

After excision, each diaphragm was quickly rinsed in Ringer's solution, the central connective tissue area was removed, and the remaining tissue was divided into 2 approximately equal parts. One half the diaphragm was rapidly weighed on a torsion balance and then placed in a Warburg flask which was gassed with oxygen for about 5 minutes. Rate of oxygen consumption was measured at 38° C during an eight hour period, with readings made at half hour intervals. All Q_{O_2} values were calculated on the basis of initial wet weight. At the end of the experiment, the tissue was weighed again, then dried in an oven at 105° C, and dry weight determined. Dry and wet weights also were obtained on the remaining half diaphragm. From these data, it was possible to estimate the total changes in water and solid content of the diaphragm maintained for the 8-hour period in the Warburg apparatus. The final concentrations of the constituents of the medium used in the Warburg flasks were as follows: NaCl, 0.11 *M*; KCl, 5.6×10^{-3} *M*; $CaCl_2$, 7.8×10^{-4} *M*; $MgSO_4$, 1.1×10^{-3} *M*; NaH_2PO_4, 1.78×10^{-3} *M*; Na_2HPO_4, 1.12×10^{-2} *M*; glucose, 2.8×10^{-2} *M*. In addition, crystalline penicillin was added to give a final concentration of about 1000 units per cc. of buffered medium. The initial pH of the

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medium was 7.4 and shifted very little during the 8 hour experiment, the maximum being a decrease of 0.10. Whenever estimates of probability are given, statistical treatment of data was by analysis of variance.

RESULTS

Table 1 summarizes the mean Q_{O_2} values and their standard deviations for each of the experimental groups. Graph A in Figure 1 presents the time course of Q_{O_2} values for tissues from normal and thyroxine pretreated animals. The mean Q_{O_2} of normal tissues rose from 2.1 to 2.3 at about 1.5 to 2 hours and then declined slowly but steadily so that by the eighth hour it was approximately one half the initial value. The mean Q_{O_2} of tissues from thyroxine pretreated animals also rose initially and then declined with time. Their wet weight Q_{O_2} values, as expected, were higher than those of the diaphragm from untreated animals at the start. However, by the third hour, the Q_{O_2} values for these two groups had become equal, and thereafter the Q_{O_2} of diaphragm from the pretreated animals, declining more rapidly, reached a level some 20% below that of the normal controls ($P < .05$) by 8 hours.

All hemidiaphragms, regardless of experimental group, lost about 28% of their dry weight during the 8 hour period and about 9% of their water content. Since all Q_{O_2} values in this report are based on the initial wet weight of the tissues and since all the tissues lost weight during the course of the experiment, the Q_{O_2} values given for the last few hours may be rather markedly underestimated and the rate of decline of Q_{O_2} (Fig. 1A) is probably not as great as indicated by the data. The more rapid rate of decline of all the tissues taken from prethyroxinized animals may reflect the more rapid destruction of some essential intracellular material. It is not caused by greater hydration or loss of weight since prethyroxinized tissues did not differ significantly from the others in these respects.

The Q_{O_2} of normal tissues exposed to thyroxine *in vitro* was followed for 8 hours in the hope that this interval would exceed the latent period and allow a stimulatory response to develop. The results are plotted (Fig. 1B) as per cent deviation from the Q_{O_2} of normal tissues not exposed to thyroxine; the untreated tissues are thus represented by the horizontal line at zero on the ordinate of the graph. Although the lower concentration of thyroxine was without significant effect, the response to the higher concentration proved to be diphasic consisting of an initial inhibition followed by a relative stimulation. Thus, the Q_{O_2} of normal tissues subjected to the higher concentration of thyroxine *in vitro* was about 10% lower than that of their controls ($P < 0.01$) for the first 2 hours, then reached control level at 3.5 hours, and for the last few hours significantly exceeded that of the controls ($P < 0.01$).

When diaphragms were taken from animals pretreated 20 hours earlier with thyroxine, the higher concentration of thyroxine *in vitro* again pro-

duced a diphasic response (Fig. 1C). The Q_{O_2} values for this group were significantly lower ($P < 0.01$) during the first 2 hours and significantly higher ($P < 0.01$) during the last 3 hours than those of the pretreated tissues not exposed to thyroxine *in vitro*. The lower concentration of thyroxine *in vitro* produced a small (about 11%) but significant ($P < 0.01$) response in pretreated tissues which was sustained over the entire 8-hour period. It should be mentioned that the only absolute stimulation found was in this group. In other groups, the stimulation was relative, being represented by a decreased rate of decline of Q_{O_2}.

DISCUSSION

Attempts to increase the rate of oxygen consumption of isolated tissues by exposure to thyroxine *in vitro* have appeared in the literature for over

TABLE 1. THE MEANS AND STANDARD DEVIATIONS OF WET WEIGHT Q_{O_2} VALUES OF HEMIDIAPHRAGMS FROM NORMAL AND THYROXINE PRETREATED RATS DURING EXPOSURE TO THYROXINE *in vitro* FOR AN EIGHT HOUR PERIOD

	Normal			Thyroxine pretreated		
Thyroxine concentration, moles/liter	0	2.0×10^{-6}	2.0×10^{-5}	0	2.0×10^{-6}	2.0×10^{-5}
No. of animals	24	12	10	24	12	12
Hours *in vitro*						
0.5	2.08±0.17	2.02±0.15	1.91±0.20	2.28±0.18	2.47±0.21	2.14±0.12
1.0	2.22±0.19	2.26±0.22	2.01±0.22	2.59±0.25	2.90±0.31	2.36±0.18
1.5	2.31±0.22	2.31±0.31	2.14±0.19	2.55±0.30	2.89±0.39	2.38±0.22
2.0	2.30±0.27	2.22±0.32	2.04±0.18	2.41±0.36	2.61±0.36	2.33±0.26
2.5	2.17±0.28	2.06±0.30	2.01±0.27	2.19±0.37	2.46±0.36	2.21±0.28
3.0	2.00±0.32	1.96±0.37	1.96±0.30	2.04±0.40	2.22±0.36	2.15±0.29
3.5	1.90±0.34	1.78±0.38	1.87±0.32	1.86±0.48	2.07±0.38	2.05±0.27
4.0	1.75±0.36	1.70±0.42	1.79±0.34	1.73±0.50	1.87±0.39	1.96±0.31
4.5	1.64±0.35	1.56±0.36	1.70±0.37	1.55±0.52	1.76±0.45	1.88±0.32
5.0	1.52±0.38	1.44±0.43	1.66±0.44	1.38±0.53	1.57±0.44	1.76±0.35
5.5	1.42±0.36	1.39±0.44	1.55±0.33	1.27±0.50	1.40±0.41	1.63±0.37
6.0	1.32±0.37	1.31±0.41	1.42±0.42	1.11±0.48	1.27±0.46	1.52±0.33
6.5	1.17±0.33	1.15±0.35	1.39±0.37	1.01±0.43	1.16±0.41	1.39±0.35
7.0	1.12±0.31	1.08±0.28	1.25±0.34	0.91±0.42	1.05±0.40	1.32±0.35
7.5	1.03±0.32	1.02±0.28	1.17±0.33	0.83±0.39	0.95±0.35	1.21±0.28
8.0	0.95±0.29	1.04±0.23	1.18±0.31	0.75±0.36	0.90±0.35	1.17±0.24

thirty years. Although more recent work (6, 7, 8) has established the effect of thyroxine on the uncoupling of phosphorylation from oxidation tion in mitochondrial preparations, the effect on rate of oxygen consumption of tissue slices or strips has remained equivocal. In a number of earlier papers (9, 10, 11) extremely low concentrations (some investigators, unfortunately, failed to mention the units used for expressing concentration) of thyroxine are reported to induce a prompt increase in the rate of oxygen consumption of isolated tissues whereas higher concentrations either had no effect or depressed metabolism. In a more recent report Wiswell *et al.* (3), studying the effect of triiodothyronine *in vitro* on isolated tissues over an eight hour period, claims that no effect was observed. However, analysis of his data over the last three hours of his experiment reveals a significant response for both diaphragm ($P < .01$) and liver ($P < .05$). His data further

suggest a slight inhibiting action during the first two hours. Weiss (4) also reports a failure of liver slices to respond to extended exposure to thyroxine *in vitro*. However, even after correcting for the decreasing Q_{O_2} of his control tissues, statistical evaluation reveals a significant initial inhibition ($P < .01$) by both thyroxine and triiodothyronine. Analysis of the data of

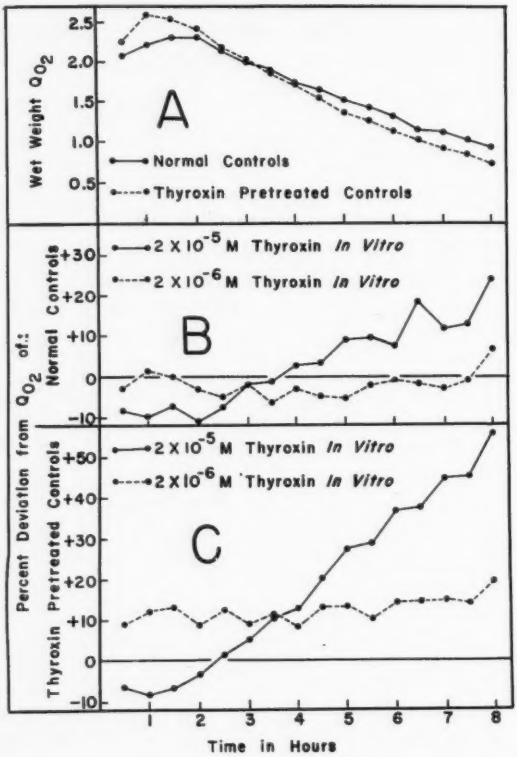

FIG. 1. Graph A shows the time course for mean wet weight Q_{O_2} of diaphragm from normal and thyroxine pretreated rats. Each point represents the mean of 24 determinations. Graph B indicates the percentage change in Q_{O_2} induced in diaphragm from normal rats by exposure to thyroxine *in vitro* over an eight hour interval. Each point represents the mean of 10 or 12 determinations (see Table 1). Graph C shows the percentage change in Q_{O_2} of diaphragm from thyroxine pretreated rats caused by thyroxine *in vitro*. Each point represents the mean of 12 determinations.

other investigators (9, 10, 11) also discloses a significant thyroxine induced depression of Q_{O_2} of isolated tissues.

A number of possible explanations for the metabolism inhibiting effect of the higher concentration of thyroxine may be considered. Conceivably, high concentrations of thyroxine are in some way "toxic" to tissues so that their metabolic rate is depressed. A more specific explanation is repre-

sented by a "two point binding theory" (12) proposed to account for the inhibiting action of high substrate concentration on the activity of certain enzymes. Both of these explanations require the inclusion of a mechanism for destruction of thyroxine in order to account for the ultimate escape from thyroxine-induced depression of Q_{O_2} and the subsequent relative stimulation.

Another possible explanation, perhaps more plausible than the preceeding, is suggested by the possibility that thyroxine is not the active form of the thyroid hormone (13). If this is the case, the "active" thyroid hormone may be competitively inhibited by thyroxine. Many compounds related to thyroxine have been shown to inhibit the action of thyroxine (14), and on the basis of the results of our experiments, thyroxine itself should be added to the list. With this theory, the ultimate relatively high Q_{O_2} of tissues exposed to the higher concentration of thyroxine may be dependent on the conversion of the thyroxine to an active form. This is an attractive possibility since it offers explanations for the latent period observed following the administration of thyroxine *in vivo*, and suggests that some of the derivatives of thyroxine which have been found to be more potent or have a shorter latent period than thyroxine itself may be related to an active form of the hormone. Thus, this theory proposes that thyroid hormone circulates in an inactive form (principally thyroxine) and after being bound to specific action sites in the cell, is converted to an active form. Then, when a tissue is exposed to relatively high concentrations of thyroxine, some of the active material may be displaced and a depressed rate of oxygen consumption may be expected to result until sufficient thyroxine has been "activated" to overcome the initial Q_{O_2} depression. The recent report by Barker (15) that kidney slices incubated at 5° C in the presence of thyroxine reveal a progressive metabolic response over a period of days suggests that kidney as well as muscle can "activate" thyroxine.

The rate of thyroxine destruction or activation (depending on which of the interpretations is considered) is apparently determined to some extent by the responding tissue. This is suggested by the finding that diaphragm from prethyroxinized animals, when exposed to the higher concentration of thyroxine, exhibits a more rapid escape from inhibition and differs more markedly from control tissues during the last few hours of the experiment than does diaphragm from normal animals. Furthermore, diaphragm from prethyroxinized animals exposed to the lower concentration of thyroxine shows a prompt and sustained response that is absent in the case of diaphragm from normal animals.

SUMMARY

The Q_{O_2} of hemidiaphragms from normal and from thyroxine pretreated rats was measured over an 8-hour period both in the presence and in the absence of thyroxine in the medium. *In vitro* thyroxine at a concentration

of 2×10^{-5} *M* significantly depressed the Q_{O_2} of both normal and thyroxine-pretreated diaphragms for about the first two hours but by the last two hours this effect had been reversed and a relative stimulation was observed. A lower concentration of *in vitro* thyroxine (2×10^{-6} *M*) had no measurable effect on normal diaphragm but produced a prompt and significant stimulation of pretreated diaphragm. It is suggested that thyroxine (1) is not the active form of the thyroid hormone, (2) competitively inhibits the active form, and (3) is converted to the active form by peripheral tissues.

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POTENTIATION BY SQUALENE OF ADRENAL AND THYMIC RESPONSES TO CORTICOTROPIN[1,2]

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SQUALENE, a triterpenoid hydrocarbon, has been identified as a naturally occurring compound which can be synthesized by animal tissues from acetate and can serve as a precursor of cholesterol, both *in vivo* and *in vitro* (1–5). The normal role of squalene as an intermediate in the biogenesis of cholesterol has not been established as being either obligatory or quantitatively significant, although there is good evidence that it is a natural precursor of cholesterol (6, 7).

Squalene inhibits the response of secondary sex organs of chicks to pregnant mares' serum gonadotropin (PMSG) (8). Since the adrenals are steroid-producing glands subject to tropic hormone control, as are the gonads, it seemed possible that squalene might also inhibit response to corticotropin (ACTH). The effect of squalene on the action of ACTH in hypophysectomized weanling male rats has therefore been studied. No inhibition of ACTH action has been observed using adrenal and thymus weights as indices of response. On the contrary, squalene augments the adrenal hypertrophy and thymus involution induced by ACTH.

PROCEDURES

Weanling male rats of the Holtzman strain, all obtained from one supplier,[4] were used exclusively. The hypophysectomized rats were operated at 21 days of age before being shipped but those subjected to adrenalectomy and castration were operated in our laboratory. The animals were housed by experimental groups on wood shavings in cages protected from drafts.

Hypophysectomized rats were fed *ad libitum* a diet of canned dog meat (Pard), milk, whole wheat bread soaked in water, lettuce, carrots, and oranges. Both intact and adrenalectomized-castrated rats were given Rockland mouse pellets. Physiological saline was supplied instead of tap water for adrenalectomized animals.

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[2] A preliminary report of some of this work was presented at the Laurentian Hormone Conference, September, 1955.

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[4] Hormone Assay Laboratories, Inc., Chicago, Illinois.

Squalene, ACTH, vehicle and other control substances were injected subcutaneously in all experiments except Experiment 51M in which squalene was administered orally to four groups (Table 1). Substances given once a day were injected just before 5:00 P.M.; oral squalene was given twice daily, at 9:00 A.M. and 5:00 P.M. No injection site was used more than once during the entire treatment period, and injection sites for different substances were widely separated.

The squalene, obtained by molecular distillation from shark liver oil, was 90–95% pure according to the suppliers.[5] Infrared absorption spectra[6] on a representative sample revealed approximately 10% contamination with a carbonyl compound which has not been identified. Because of poor absorption from subcutaneous depots, each day's total dose of squalene (0.2 ml.) was divided into four equal amounts of 0.05 ml. and injected at four different but closely adjacent sites widely removed from the sites of injection of other substances. Squalene from the same lot was used in all experiments.

In the hypophysectomized rats (Exp. 51M, Table 1), the treatment period was of 7 day's duration, beginning on the 27th day of age. Squalene was given daily while ACTH was injected only the last five of these seven days, as 0.075 ml. (1.5 Armour Units) of H P ACTHar gel per day. This schedule allowed 6 days for adrenal regression prior to the beginning of squalene injections and 8 days prior to ACTH treatment.

In the experiments employing intact or adrenalectomized rats, the treatment periods were also of 7 days' duration. The intact rats in Experiment 70M (Table 2), were injected subcutaneously daily from the 23rd through the 29th days of age with squalene, mineral oil (U.S.P. Heavy), olive oil (Grandee Brand), or erucic acid (practical grade, m.p. 24–27° C). Each substance was given to two groups of rats in daily doses of 0.2 or 0.4 ml., using 0.05 ml. per injection site. The adrenalectomized-castrated rats, were operated at 28 to 29 days of age and those which received squalene were injected daily in the amount of 0.05 ml. at each of four sites, from 30 through 36 days of age.

Autopsies were performed 16 to 24 hours after the last injections in all experiments. The animals were killed quickly with chloroform; adrenals and thymi were removed, freed of extraneous tissue, and weighed promptly on a Roller-Smith torsion balance.

RESULTS

Squalene given by subcutaneous injection clearly augmented adrenal response to ACTH with respect to both growth and cortical function in hypophysectomized rats under the conditions of Experiment 51M (Table 1), selected as a representative experiment. Simultaneous squalene and ACTH treatment resulted in 25% heavier adrenals and 50% smaller thymi, than did treatment with ACTH alone. The amount of ACTH given was selected to produce significant but far from maximal changes in adrenal and thymus weights so that measurement of augmentation was possible without attaining the maximal effect known to occur with high doses of ACTH alone. Squalene itself did not affect adrenal and thymus weights, nor did gelatin, saline or sham injections. The uniformity of all these control groups is reassurance that conditions of housing, feeding and handling were consistent from cage to cage. Squalene given orally in the same amount as was injected, or in even twice that amount, did not

[5] Distillation Products Industries, Rochester, New York.

[6] We are indebted to Drs. R. I. Dorfman and H. Rosenkrantz, The Worcester Foundation for Experimental Biology, for the infrared spectra.

significantly augment ACTH action on the adrenals with respect to either glandular weight or function, although the changes in adrenal and thymus weights which did occur were consistently in directions expected for such an effect.

The effect of squalene in intact rats was tested in an experiment in which three other substances were similarly administered as control materials. The results are presented in Table 2. Squalene, mineral oil and olive oil did not change adrenal or thymic weights. Erucic acid, however, did cause adrenal enlargement and thymic involution in proportion to dosage.

In adrenalectomized-castrated rats (Table 3) injected for 7 days with 0.2 ml. of squalene per day, the absolute and relative thymus weights were the same as those of uninjected adrenalectomized-castrated controls.

DISCUSSION

Use of thymus involution as an indicator of adrenocortical response to experimentally imposed conditions combines the advantages of measuring the functional activity of the gland, of making this measurement simply, and of permitting simultaneous assay of ACTH effectiveness by adrenal weight increase.

Acute involution of the thymus is now recognized as a specific and quantitative response to glucocorticoids even in intact rats and mice (15), but only under conditions which exclude effects from certain other steroids, x-irradiation, general debilitation, and toxic substances, such as urethane, benzene, and nitrogen or sulfur mustards (9–11). All of these factors can induce thymic involution not only indirectly by activation of the pituitary-adrenal axis, but also directly, without the intervention of the adrenal. Even conditions of stress can exert a direct effect on thymic tissue conditioned with corticoids (11). In addition, testosterone, estradiol, progesterone, and deoxycorticosterone potentiate cortisone in its thymolytic action in adrenalectomized-ovariectomized mice (12). In Experiment 51M re-

TABLE 1. POTENTIATION OF ACTH BY SQUALENE IN HYPOPHYSECTOMIZED RATS

Treatment	Autopsy data: Mean values ± S.E.*			Statistical analysis: P values		
	Body weight, gm.	Thymus, mg./100 gm. body weight	Adrenals, mg./100 gm. body weight	Compared to:	Thymus	Adrenals
Untreated controls	59.4±2.9	251.7±20.2	12.9±0.6	—	—	
Squalene (S.C.)	65.9±1.6	249.0±16.7	14.7±0.7	Controls	—	0.1
ACTH	62.0±1.4	170.8±10.7	19.7±1.5	Controls	0.01–0.001	0.001
ACTH & squalene (S.C.)	57.4±1.4	90.5± 9.5	25.3±2.0	ACTH	0.001	0.05–0.02
Gelatin (10%)	61.5±2.9	242.6±23.0	14.0±1.0	Controls	0.5	0.4
Saline (0.9%)	61.5±0.7	235.5±19.8	13.8±0.6	Controls	—	0.4
Sham injections	62.1±2.3	274.7± 8.7	12.0±0.7	Saline	0.1	0.1
Squalene (oral, 0.2 ml./day)	63.5±1.8	235.5±19.8	13.6±0.5	Controls	No sig. difference	
Squalene (oral, 0.4 ml./day)	63.9±1.3	268.3±11.6	13.1±0.1	Controls	No sig. difference	
ACTH & squalene (oral, 0.2 ml./day)	61.6±2.2	144.5±12.1	20.3±1.0	ACTH	No sig. difference	
ACTH & squalene (oral, 0.4 ml./day)	60.4±1.8	152.5±12.5	21.5±1.6	ACTH	No sig. difference	

* Standard error of the mean. Eight rats per group.
S.C. Subcutaneous injection.

TABLE 2. FAILURE OF SQUALENE TO AFFECT THYMUS OR ADRENAL WEIGHTS IN INTACT RATS

Treatment	Autopsy data¶: Mean values ±S.E.§			
Substance injected	Daily dose, ml.	Body weight, gm.	Thymus, mg./100 gm. of body wt.	Adrenals, mg./100 gm. of body wt.
None (Initial controls)	0.0	52.4±1.2	333.2±13.8	38.8±1.8
None (Final controls)	0.0	86.1±2.9	344.6±19.1	24.7±1.6
Squalene	0.2	87.8±2.7	337.3±14.4	25.0±0.9
Squalene	0.4	86.6±1.6	314.8±12.7	27.6±0.8
Mineral oil	0.2	93.3±2.0	333.5±18.9	24.5±0.5
Mineral oil	0.4	91.9±2.2	350.4±12.6	23.5±1.0
Olive oil	0.2	84.8±2.0	369.0±18.7	25.3±0.5
Olive oil	0.4	87.1±1.5	361.5±13.2	26.2±0.8
Erucic acid	0.2	82.3±2.0	**268.5±23.8	*31.3±1.3
Erucic acid	0.4	*68.5±2.0	* 48.1± 8.5	*38.1±1.6

§ Standard error of the mean.
¶ Eight animals per group. Initial controls autopsied at 23 days of age; other groups, at 30 days of age.
* Difference from final controls statistically significant, $P < 0.001$.
** Difference from final controls statistically significant, $P = 0.05 - 0.02$.

ported here, the use of hypophysectomized animals maintained under optimal conditions has obviated thymolytic factors, both indirect and direct, other than those experimentally imposed. Evidence in support of this claim is that an increase in body weight which occurs after hypophysectomy in young male rats on well balanced and palatable diets (13), occurred in our animals.

Several investigators have demonstrated a linear relationship between the log dose of corticoids, or the log dose of ACTH, and the relative thymus weight in adrenalectomized or intact rats and mice (14–17). Thymic regression in intact mice has been used to quantitate corticoid secretion in response to the stress of an intoxicating dose of alcohol (18). The adrenal steroids effective in causing thymus involution are those with an oxygen function at C-11 and their potencies in this regard increase progressively with the following functional groups or combinations of functional groups: 11-ketone, 11β-hydroxyl, 11-ketone plus 17α-hydroxyl, and 11β-hydroxyl plus 17α-hydroxyl (15).

If, then, thymic involution under the conditions used in Experiment

TABLE 3. FAILURE OF SQUALENE TO AFFECT THYMUS WEIGHT IN ADRENALECTOMIZED RATS

Treatment	No. of rats	Autopsy data: Mean values S.E.*		
		Body weight, gm.	Thymus weight, mg.	Thymus, mg./100 gm. body weight
None	13	91±3.0	401±20.8	441±16.6
Squalene **	12	87±3.5	386±20.0	443±12.5

* Standard error of the mean.
** 0.2 ml. per day for 7 days, injected subcutaneously.

51M (Table 1) is an acceptable index of adrenal production of glucocorticoids, it appears that hypophysectomized rats injected with squalene are able to respond to a given dose of ACTH with an elaboration of corticoids either qualitatively more potent or quantitatively greater in amount, or both, than can ACTH-treated rats not supplemented with squalene. Chronic treatment of rabbits with ACTH for seven days is known to change the corticoid secretion (19) from corticosterone to a mixture of corticosterone and 17-hydroxycorticosterone in a ratio of 2:1. Such a change might also occur in the rat adrenal in response to such ACTH stimulation, since the rat typically secretes corticosterone almost exclusively (20).

The failure of orally administered squalene to augment ACTH is in contrast to the potentiation exerted by subcutaneously injected squalene. This is due perhaps to the even poorer than usual absorption of squalene in hypophysectomized rats in which the characteristically depressed intestinal function has not been restored completely to normal by the less than optimal dose of ACTH administered. That the 0.4 ml. oral dose of squalene is no more effective than the 0.2 ml. dose seems consistent with the ideas that intestinal absorption mechanisms are already saturated at the lower dose or that hepatic utilization is not maximal at the higher dose.

The possibility of subcutaneously injected squalene having a direct thymolytic action was explored in adrenalectomized-castrated rats even though squalene alone failed to cause thymic regression in hypophysectomized rats. The data (Experiment 62M, Table 3) show that squalene did not interfere with thymic growth, which was presumably occurring at a maximal rate in the absence of its major normal deterrents, the adrenal and gonadal steroids (15).

That squalene might activate the pituitary-adrenal axis or potentiate endogenous ACTH in intact rats was investigated (Table 2, Exp. 70M). During a 7-day period at an age when the thymus, but not the adrenal, was still growing in proportion to the body, squalene was injected subcutaneously as in the experiments with hypophysectomized rats. The same dose or even twice the dose of squalene, which potentiated exogenous ACTH in the hypophysectomized rats, did not produce adrenal enlargement or thymus involution in the intact rats. There is no evidence, therefore, of increased ACTH secretion nor of agumentation of endogenously secreted ACTH. Either these effects did not occur or they were within a range in which compensation by homeostatic mechanisms was possible.

In this same experiment (Table 2), three control substances were also tested in intact rats,—mineral oil, olive oil and erucic acid. Mineral oil was selected because it is not absorbed. Its failure to cause thymic involution ruled out an anticipated but not realized possibility that squalene, being poorly absorbed from subcutaneous depots, could exert an indirect thymolytic effect through mechanical irritation of the skin. Olive oil is an

easily absorbed fat which, when fed as 25% by weight of the diet for 4 weeks, has been shown to double the adrenal cholesterol of intact rats (21). In our groups injected with olive oil, any increase in adrenal cholesterol which might have occurred was not a trigger for uncompensated increased adrenocortical secretion. Erucic acid has been identified as the component of rapeseed oil mainly responsible for the three- to five-fold increase in adrenal cholesterol which occurs in intact rats fed this oil as 25% by weight of the diet for 4 weeks. Feeding erucic acid as 15% of the diet for 3 weeks gives a similar response (21–23). In our experiments, subcutaneous injection of erucic acid resulted not only in significantly increased adrenal weight but also in extreme thymic involution. Since the erucic acid was injected without purification and without being neutralized, these effects may have various explanations. The results do provide, however, a contrast which emphasizes the innocuousness of injected squalene and illustrates an effect beyond the control of homeostatic mechanisms.

Despite the failure of squalene to cause thymic involution directly in hypophysectomized or adrenalectomized animals, the possibility has not been ruled out that an effect of squalene might be demonstrated in animals with impaired hormonal homeostatic mechanisms if the thymic tissue were conditioned by corticoid treatment; that is, the permissive role of corticoids might become apparent in the absence of hormonal homeostatic mechanisms (24). This matter is under investigation and results will be reported elsewhere.

Squalene has not augmented ACTH action in every experiment done with hypophysectomized rats and the degree of augmentation by squalene has varied from experiment to experiment. Equivocal results of early experiments are now interpreted as due to differences in dosages and techniques of injection of squalene, to differences in treatment schedules, or to inadequate attention to the food of the animals. Standardization of animal care and of treatment schedules has produced more uniform results. Several parameters remain to be studied, however, and investigation of their relations to the response to ACTH and squalene, individually or combined, may provide an insight into the reason for the failure of squalene to augment ACTH action in about one out of four experiments. Inspection of the data accumulated to date gives the impression that augmentation of ACTH action by squalene occurs with doses of ACTH which alone cause 13–32% decrease in thymus weight, and is maximal with a dose of ACTH which produces a 20–25% decrease in relative thymus weight. Squalene may become limiting with the higher doses of ACTH.

Augmentation by squalene of ACTH-induced increase in adrenal weight is difficult to understand but would be most simply and obviously explained by accumulation of squalene or cholesterol in the gland. Preliminary results indicate, however, that cholesterol content does not account for the increased adrenal weight. The squalene content of the adrenals has not been

determined. Adrenal hypertrophy or hyperplasia, it seems, could occur only if squalene, by a sparing action with reference to one or more of the postulated multiple functions of ACTH, increased the amount of ACTH available for promoting protein synthesis in adrenal tissue.

The data reported in this paper are indirect evidence consistent with the idea that squalene augments ACTH action because the hydrocarbon enters the reaction sequence of adrenal corticoid synthesis in such a way as to circumvent a rate-limiting step. Evidence of a more direct type will be required, however, to determine whether or not squalene (or a closely related compound) can be and is normally converted biologically to corticosteroids and, if such a conversion does occur, whether or not cholesterol is involved as an intermediate, obligatorily or otherwise.

It is not readily apparent why squalene should augment response to ACTH in hypophysectomized rats and inhibit response to PMSG in normal chicks. If squalene is to be regarded as a precursor of adrenal corticoids in explaining increased response to ACTH, one would predict that squalene should augment instead of inhibit response to PMSG in cockerels since cholesterol, and therefore presumably squalene, can serve as a precursor of C_{19} steroids (26, 27). Estrogens, on the other hand, do not arise from cholesterol according to all existing evidence (25). Conceivably, therefore, inhibition of estrogenic effects by squalene might occur at the biosynthetic level. Reconciliation of these complexities with reference to the effects of squalene, observed as they have been in two different species and in animals differing in physiological status, will be possible only after further investigation.

SUMMARY

Squalene injected subcutaneously for seven days augments the effectiveness of ACTH as measured by adrenal hypertrophy and thymus involution in hypophysectomized, weanling male rats. The augmentation occurs with doses of ACTH which alone cause 13–32% decrease in relative thymus weight. Squalene *per se* has no effect in hypophysectomized, adrenalectomized or intact rats under the experimental conditions used.

The data are consistent with the idea that exogenous squalene can serve *in vivo* as a readily available, easily utilized precursor of glucocorticoids and support the hypothesis that squalene is a natural intermediate in steroid biogenesis.

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ADDENDUM

After this article had been submitted for publication, evidence that biosynthesis of estrone occurs in major part from acetate by way of cholesterol was presented by K. Savard *et al.* (*Fed. Proc.* **16:** 241. 1957). Inhibition by squalene of the response of secondary sex organs of both sexes to PMSG will presumably, therefore, be found to be explained by the same or very similar mechanisms.

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ABSORPTION AND SECRETION OF IODIDE BY THE INTESTINE OF THE RAT[1]

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THE study of the absorption of iodide by the intestinal tract was initiated by Cohn (1), who showed that iodide was well absorbed by the ileum, jejunum and colon of the dog, and poorly by the stomach. Albert *et al.* (2) found that the rat also absorbed iodide well from the small and large bowel and only slightly from the stomach. Pearlman, Morton and Chaikoff (3) demonstrated in the rat that the tissue of the entire small intestine accumulated at the end of one hour 0.6% of the I^{131} administered into the stomach. The studies of Johnson and Albert (4) revealed I^{131} in the contents of the small intestine of the rat in significant concentrations, but they did not establish whether it was gastric iodide being passed down the intestinal tract or actively secreted at that site. Furthermore, Honour *et al.* (5) concluded that while the iodide concentration of stomach secretions was thirty times that of plasma, the iodide concentration of duodenal secretions was approximately equal to that of plasma.

Bearing in mind the demonstration by Kremin *et al.* (6) that removal of the distal but not the proximal one-half of the small bowel of the dog greatly interfered with fat absorption, a further investigation of the absorption and secretion of iodide by various segments of the intestinal tract was undertaken.

EXPERIMENTAL

Male albino rats weighing 200–300 gm. were used in this study. Each animal was anesthetized with pentobarbital, the abdominal cavity was opened, the bile duct was tied, and ties were placed around the intestines dividing the small intestine into five or six separate segments (consecutively numbered 1 to 5 or 6) and the large intestine into cecum and two other segments. The ties were placed so as to interfere with blood supply as little as possible. One microcurie of I^{131} in 0.1 cc. was injected into the lumen of each bowel segment. The dose of I^{131} when given subcutaneously was 50–80 μc. In some animals sodium perchlorate was injected subcutaneously 30 minutes before the bowel was tied. Those animals receiving propylthiouracil were injected subcutaneously 90 minutes before the bowel was tied. One hour after the tracer was injected the animals were sacrificed and the unopened bowel segments removed, placed in paper cups and

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counted at a distance of 22 cm. from a scintillation crystal. The bowel segments were then weighed with their contents. In a number of animals the small intestines were washed free of their contents with 50 cc. of saline that was injected through the pylorus and allowed to run out through an incision in the terminal ileum. The remaining saline was milked out. The bowel was then ligated and I^{131} injected subcutaneously. After the segments were weighed and counted, they were opened, washed free of accumulated secretions, blotted, reweighed, and recounted.

RESULTS

To establish the ability of various isolated intestinal segments to absorb iodide, I^{131} was injected into their lumina. Table 1 shows the percentage of the injected dose remaining in each segment after one hour. In two segments iodide was slowly removed. One of these was just beyond the middle of the small intestine, and the other was the cecum. The other areas absorbed iodide quite rapidly.

TABLE 1. THE PER CENT OF ADMINISTERED DOSE OF I^{131} LEFT IN THE INTESTINAL SEGMENTS AT THE END OF ONE HOUR. THE RANGE OF VALUES OF EACH SEGMENT IS FOR THREE RATS

Duodenum	Small intestine				Cecum	Colon	
1	2	3	4	5	6	7	8
8–13	9–36	16–27	74–86	33–40	48–84	16–25	13–16

TABLE 2. THE AMOUNT OF I^{131} ACCUMULATED IN ISOLATED INTESTINAL SEGMENTS AND THE EFFECT OF 10 MG. OF SODIUM PERCHLORATE ON THIS ACCUMULATION. UNITS ARE THE FRACTIONAL RADIOACTIVITY ACCUMULATED IN EACH SEGMENT DIVIDED BY THE FRACTIONAL WEIGHT OF EACH SEGMENT[1]

Drug	No. rats	Duodenum	Small intestine					Cecum	Colon	
		1	2	3	4	5	6	7	8	9
A. ———	8	1.75	0.93	1.73	2.30 ± .222*	1.05	.53	.40	.77	.71
B. perchlorate	6	1.66	1.42	.89	1.36 ± .200*	.97	.88	.72	1.58	1.08

* = S.E. of mean (reported only where a significant difference existed).
[1] See text footnote 2.

To measure the rate of secretion of iodide by various segments of the intestine, radioiodine was injected subcutaneously after the ligatures had been placed. The values were expressed as the fractional radioactivity accumulated in each segment divided by the fractional weight of each segment.[1] Thus if the secretion of each segment were the same, each value would equal one. Table 2 shows that the accumulation of iodide was most rapid near the center of the small intestine and also quite rapid in the duodenum. The accumulation in the cecum, on the other hand, was slow.

The administration of sodium perchlorate prior to the injection of I^{131}

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[2] $\dfrac{\text{Radioactivity in segment}}{\text{Total radioactivity in bowel}} \div \dfrac{\text{Weight of segment}}{\text{Total weight of bowel}}$

TABLE 3. THE RATIO OF THE CONCENTRATION OF I^{131} IN ONE GRAM OF INTESTINAL SECRETIONS (S) AND INTESTINAL TISSUE (I) TO THE CONCENTRATION OF I^{131} IN ONE CC. OF PLASMA (P). VALUES ARE THE MEAN VALUES OF 5 RATS

Small intestinal segment	1	2	3	4	5
S/P	.85 ± .13*	1.84 ± .68*	8.60 ± 2.22*	4.74 ± 1.55*	1.98 ± .89*
I/P	.35 ± .045*	.44 ± .067*	.85 ± .155*	.53 ± .099*	.25 ± .032*

*=S.E. of mean.

caused the accumulation of I^{131} by the central segment of the small intestine to fall to about three-fifths of the control value. This is shown by Table 2. In this type of experiment perchlorate had no demonstrable effect on reducing accumulation of iodide by any other of the intestinal segments.

In order to measure the concentration of iodide in intestinal secretions uncontaminated by food, the small intestines were washed out with saline, the saline milked out and the ligatures placed. I^{131} was then injected subcutaneously, and one hour later the weight of each segment, the weight of its secretions, the radioactivity of the secretions, and the radioactivity of the intestinal wall were determined. In addition, the radioactivity of plasma samples was obtained. That iodide was actually secreted into the lumen of the small intestine and secreted by the central segments in concentrations in excess of that of plasma is shown by Table 3. The secretion of I^{131} by segment 3 (reflected in the S/P ratio) was significantly greater than that of segments 1, 2, and 5. Furthermore, the accumulation of I^{131} in the wall of segment 3 (reflected in the I/P ratio) was significantly greater than that of segments 1, 2, and 5.

Finally, 5 mg. of propylthiouracil was given to inhibit organic binding of iodide. A comparison of the data of Tables 3 and 4 shows that it had no significant effect on iodide secretion by the small intestine. Perchlorate reduced the entry of iodide into the lumen of all the segments to a uniform level, and also lowered the amount of iodide accumulated in the wall of these segments to a uniform level.

TABLE 4. THE RATIO OF THE AMOUNT OF I^{131} IN ONE GRAM OF INTESTINAL SECRETIONS (S) AND INTESTINAL TISSUE (I) TO THE AMOUNT OF I^{131} IN ONE CC. OF PLASMA (P) IN ANIMALS PRETREATED WITH PROPYLTHIOURACIL, AND THE EFFECT OF SODIUM PERCHLORATE ON THIS RATIO

	Drug	No. rats	Small intestinal segment				
			1	2	3	4	5
S/P	—	5	1.08 ± .17*	2.39 ± 1.21	5.12 ± .74	2.44 ± .57	1.34 ± .29
S/P	$NaClO_4$	6	.64 ± .07	.66 ± .07	.76 ± .11	.68 ± .12	.55 ± .09
I/P	—	5	.31 ± .03	.99 ± .26	1.81 ± .40	.77 ± .20	.52 ± .10
I/P	$NaClO_4$	6	.21 ± .03	.24 ± .05	.19 ± .02	.16 ± .02	.19 ± .02

*=S.E. of mean.

DISCUSSION

Iodide was not uniformly absorbed by the small and large intestine, but was relatively poorly absorbed in the middle of the small intestine and in the cecum. This effect was apparently masked by the rapid uptake of nearby intestinal tissue in the earlier study of Albert *et al.* (2).

The ability of the mid-portion of the small intestine to secrete iodide is shown by Table 2. This portion behaves like the stomach which absorbs iodide poorly although it secretes it rapidly (1, 2). Clearly the cecum does not fit this pattern, since it secretes iodide as poorly as it absorbs it. This poor absorption may be due to poor mixing of the bulky cecal contents as the cecum had little observable motility. The ability to secrete iodide tapered off on each side of the middle segment of the small intestine.

The data of Table 3 show that the accumulation of iodide in isolated intestinal segments had two components. Although most of the iodide was secreted into the lumen of the intestine, a small amount remained in the intestinal wall. That segment which had the greatest secretory ability also had the largest amount of iodide in its wall.

Perchlorate has been shown to be a potent inhibitor of the iodide-concentrating mechanism of the thyroid (7), salivary glands in man (8) and stomach (9). Perchlorate also inhibits the iodide-concentrating mechanism of the small intestine, reducing the iodide transport of not only the central segments but all segments to a uniform level. This was not shown by the data in Table 2 because of the variations in weight produced by varying amounts of feces in the bowel segments. The entry of iodide into the intestine after the administration of perchlorate can be accounted for on the basis of diffusion.

It has been demonstrated in man that 15 to 20% of Meckel's diverticula have areas of ectopic gastric mucosa (10). Since these areas secrete acid and behave as gastric mucosa, it would seem reasonable that they might secrete iodide. However, no evidence of a Meckel's diverticulum in the rat could be found. Histological sections of this new iodide secretory site were prepared, but no difference in structure from other sites could be found with a hematoxylin and eosin stain.

SUMMARY

The ability of the rat intestine to absorb and secrete iodide has been studied. It has been shown that not only does the mid-portion of the small intestine absorb iodide poorly, but it also secretes it rapidly. This is accomplished by a specific secretory mechanism that is possessed to a lesser extent by the rest of the small intestine and is similar to that already described for stomach and thyroid and salivary glands of man.

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FATE OF ADRENAL ASCORBIC ACID: RELATIONSHIP TO CORTICOSTEROID SECRETION[1]

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SINCE the classical demonstration by Long and Sayers and their co-workers (cf. 1–3) that adrenal ascorbic acid levels were acutely depleted under conditions of enhanced adrenal cortical activity, the fate of the vitamin disappearing and its role in the production, secretion and utilization of the corticosteroids have been the subject of extensive inquiry (cf. 4, 5). Only Vogt (6) appears to have investigated directly the possibility that the ascorbic acid lost by the adrenal gland under these circumstances might be secreted into the adrenal vein. However, she was unable to demonstrate a consistent secretion of this vitamin into the adrenal venous effluent of the dog even after splanchnic stimulation. The release of ascorbic acid into the perfusion fluid by adrenal glands *in vitro* has been noted by Heard and Welch (7) and Rosenfeld (8). Peripheral plasma levels of ascorbic acid have been observed to rise following prolonged bleeding (1) and splanchnic stimulation (9) in the rat and ACTH administration in man (10). However, the contribution of the adrenal gland to this increase has not been determined.

Depletion of adrenal ascorbic acid in the rat occurs predominantly within the first 30 minutes following acute activation of the adrenal cortex and is followed by a fairly rapid return to normal levels (1–3, 11). This early period, therefore, would appear to be the proper time to determine whether the vitamin disappearing can be detected in the venous effluent of the adrenal gland. The results presented below do in fact demonstrate that the ascorbic acid lost by the adrenal gland within the first 30 minutes following cannulation of the renal vein in the intact rat and following cannulation and ACTH administration in the hypophysectomized rat could be recovered quantitatively in the adrenal venous effluent. The release of ascorbic acid preceded the active secretion of corticosteroid by the adrenal gland.

METHODS

Adult male Sprague-Dawley rats weighing 200–300 gm. were employed in these studies. Normal animals were obtained from our breeding colony and were maintained

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on Purina Laboratory Chow and tap water. Hypophysectomized rats were purchased from Hormone Assay Laboratories, Chicago, Ill. The latter were fed a supplement of 5% glucose in drinking water from the time of arrival and were used 3 days post-operatively. All animals were kept in individual cages in a temperature-controlled room (78°F) prior to experimentation.

Laparotomy was performed on animals anesthetized with Nembutal (pentobarbital sodium). Nembutal appears to have little or no stimulatory effect on the pituitary-adrenocortical system (12). Approximately 0.5 ml. of blood was immediately withdrawn by syringe from the dorsal aorta at its juncture with the adrenal arteries. This blood sample was used for the determination of ascorbic acid in the systemic circulation prior to stress or ACTH injection. The left kidney was excluded from the circulation by a ligature and the left renal vein was then cannulated and ligated at its juncture with the vena cava. Preceding ligation, the animal was given ca. 0.2–0.3 mg. of heparin via the cannula.

The right adrenal gland was rapidly removed for the estimation of "pre-collection" levels of adrenal ascorbic acid. At this time, hypophysectomized rats were given ACTH (0.5 I.U. per 100 gm.)[2] via a branch of the right femoral vein. The entire operative procedure required an average of 12 minutes (11 to 13 minutes). Collections of the adrenal venous effluent were made for a total elapsed time in different animals of 15, 30, 45 or 60 minutes following cannulation. Collecting tubes were kept chilled in an ice bath. The volumes of venous blood obtained were measured and aliquots were analyzed for ascorbic acid and corticosterone content. Some of the collections were made under nitrogen, but the values obtained were not different from those found when this precaution was omitted. It has been shown earlier that blood total ascorbic acid is not quantitatively altered by contact with red cells for periods in excess of 1 hour (10). At the termination of the collection period, 0.5 ml. of blood was again taken from the dorsal aorta for the estimation of "post-collection" levels of circulating ascorbic acid. The left adrenal gland was also removed at this time and analyzed for the vitamin. In a number of similarly-treated normal and hypophysectomized animals, ascorbic acid concentrations in the dorsal aorta were followed consecutively in 0.1 ml. blood samples withdrawn at 15 minute intervals throughout the entire collection period.

Analyses for whole blood and adrenal ascorbic acid were performed using, where required, a micro-modification of the method of Roe and Kuether (13). The maximum % error of these determinations was less than 2% (for 6 replicate determinations on the same sample of blood). In a number of control studies, ascorbic acid analyses of right and left adrenals obtained from the same intact or hypophysectomized rat revealed no significant difference in the concentration of this vitamin in the two glands (cf. also 11). It was assumed, therefore, that the level of ascorbic acid in the left adrenal gland at cannulation was the same as that in the right adrenal removed for analysis at that time.

Comparison of aortal and adrenal venous levels of ascorbic acid required the assurance that hemoconcentration or hemodilution had not occurred upon passage of blood through the adrenal gland. Accordingly, hemoglobin analyses were performed by the alkaline hematin method on a number of representative blood samples. No significant differences in hemoglobin concentration were found between arterial and venous blood. However, there was a tendency for slight hemodilution (ca. 5%) to occur over the longer periods of observation (45 to 60 minutes post-cannulation), presumably due to the slow loss of blood via the cannulated adrenal vein.

Corticosterone in adrenal venous blood was measured as described earlier (12).

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[2] The ACTH employed in these studies was generously provided by the Armour Laboratories, Chicago, Illinois (ACTHAR, 1.75 I.U. per mg.).

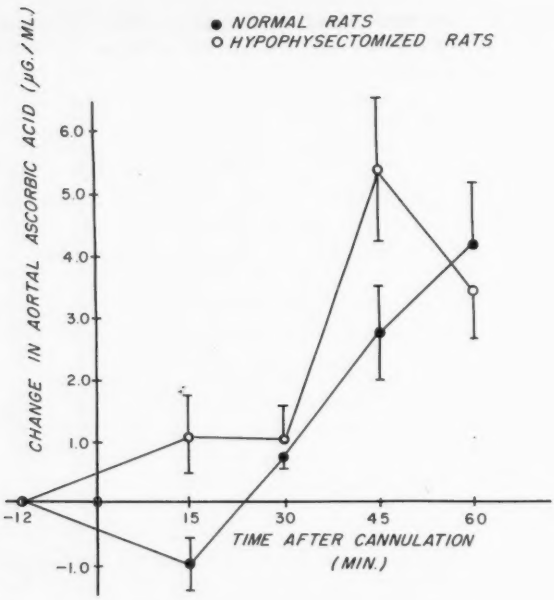

FIG. 1. Extra-adrenal influences on aortal ascorbic acid levels in normal rats subjected to stress and hypophysectomized rats given 0.5 I.U. ACTH per 100 gm. body weight at 0 time. The mean values and the standard errors (T-shaped bars) are shown for 5 to 7 rats in each group at the time of laparotomy (−12), cannulation (0) and at 15 minute intervals thereafter.

The method consisted essentially of extraction of the blood samples by the method of Bush (14) as modified by Singer and Stack-Dunne (15), and chromatography of the purified extract on filter paper (cf. 12). The filter paper was then sprayed with alkaline blue tetrazolium. The stained material possessing the chromatographic mobility of corticosterone was eluted and estimated photometrically by a micro-modification (16) of the procedure described by Touchston and Hsu (17).

RESULTS AND DISCUSSION

Aortal ascorbic acid concentrations were not significantly altered for 30 minutes following cannulation of the renal vein in either normal rats or in hypophysectomized rats given ACTH (Fig. 1). Initial levels (−12 min.) averaged $1.05 \pm .02$ mg./100 ml. in 24 normal rats and $0.91 \pm .05$ mg./100 ml. in 25 hypophysectomized animals. These values rose rapidly over the later periods following cannulation, reaching an elevation of approximately 50% within 1 hour. Ascorbic acid concentrations in micro aortal samples from animals bled at all time intervals were similar to those obtained in rats sampled only at the beginning and end of the experiment. The data presented below were obtained in the latter animals where the only major blood loss was via the cannulated renal vein.

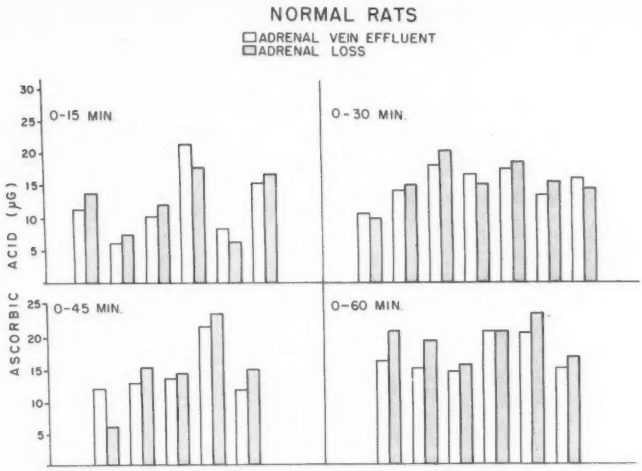

FIG. 2. Recovery of ascorbic acid disappearing from the adrenal gland of normal rats subjected to stress in the adrenal venous effluent. Each pair of bars represents data obtained on one animal. The interval following cannulation of the adrenal vein is shown in the upper left-hand corner of each segment of the chart.

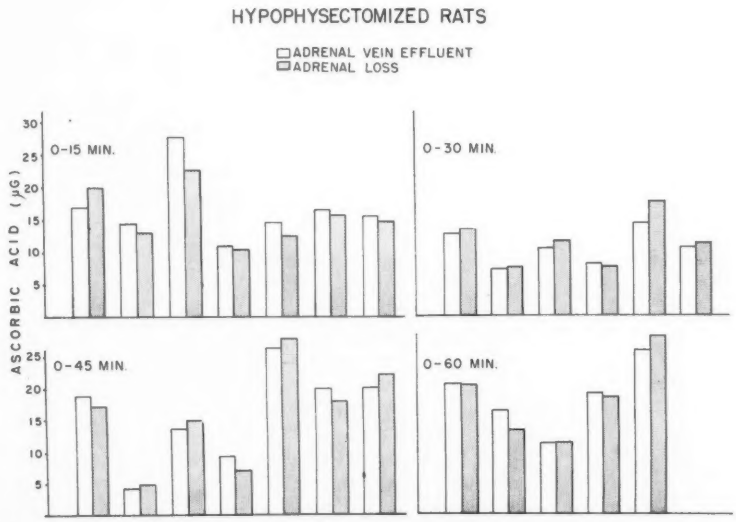

FIG. 3. Recovery of ascorbic acid disappearing from the adrenal gland of hypophysectomized rats given ACTH in the adrenal venous effluent. Each pair of bars represents data obtained on one animal. The interval following cannulation of the adrenal vein and intravenous injection of ACTH (0.5 I.U. per 100 gm. body weight) is shown in the upper left-hand corner of each segment of the chart.

It should be emphasized that the adrenal glands could not have contributed to circulating ascorbic acid levels, since the right adrenal was removed at zero time (Fig. 1) and the venous output of the left adrenal was quantitatively collected. For the same reasons, changes in circulating levels of ascorbic acid were independent of adrenal hormonal activity. The major source of the additional ascorbic acid appearing in the systemic circulation under these circumstances may have been the liver (cf. 1). It is apparent that changes in blood ascorbic acid levels (cf. 1, 9, 10) cannot be considered a reliable index of enhanced adrenocortical activity or of adrenal ascorbic acid depletion resulting therefrom.

Depletion of adrenal ascorbic acid occurred principally within the first 15 to 30 minutes following the severe stress of laparotomy in normal rats (Fig. 2, stippled bars) or the administration of a large dose of ACTH to hypophysectomized animals (Fig. 3, stippled bars). Similar results have been reported under comparable conditions by Long (1) and Sayers *et al.* (11). Quantitative recovery in the adrenal venous effluent of the ascorbic acid lost by the adrenal gland is also shown in Figures 2 and 3 (open bars). Calculations of ascorbic acid secretion by the cannulated adrenal were

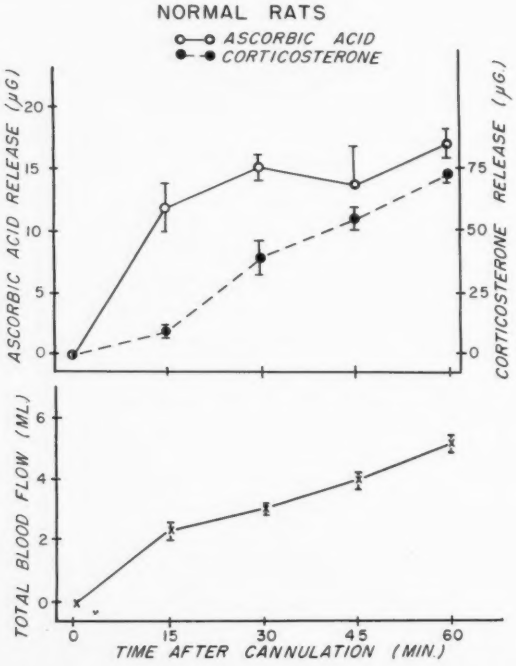

FIG. 4. Comparison of total release of adrenal ascorbic acid and corticosterone into the adrenal venous effluent of normal rats subjected to stress. Adrenal venous blood flow is also graphed. The means and standard errors are shown for 5 to 7 rats at each point

made by subtracting the average aortal ascorbic acid level over the entire period studied from the concentration of this vitamin in the adrenal venous effluent. The average arterio-venous difference obtained was then multiplied by the volume of the effluent for the period of collection. Adrenal ascorbic acid secretion values for the 15 and 30 minute periods of collection of adrenal venous effluent may be considered accurate in both the normal rats subjected to stress (Fig. 2) and the hypophysectomized animals given ACTH (Fig. 3), in view of the fact that aortal ascorbic acid concentrations were relatively stable over this period of time (Fig. 1). On the other hand, the rapidly rising arterial levels of this vitamin after 30 minutes place considerable doubt on the reliability of the average value employed to calculate arterio-venous differences in the 45 and 60 minute postcannulation groups. Nevertheless, the correspondence of these differences with the actual amounts of ascorbic acid lost by the adrenals was remarkably good (Figs. 2 and 3).

The acute release of ascorbic acid into the adrenal venous effluent under circumstances of enhanced adrenal cortical activity was essentially com-

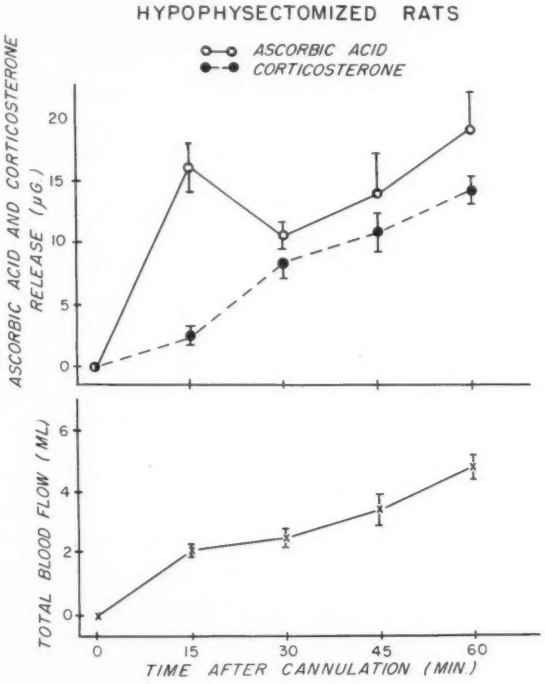

FIG. 5. Comparison of total release of adrenal ascorbic acid and corticosterone into the adrenal venous effluent of hypophysectomized rats injected intravenously with 0.5 I.U. ACTH per 100 gm. body weight. Adrenal venous blood flow is also graphed. The means and standard errors are shown for 5 to 7 animals at each point.

plete within 15 minutes following cannulation in normal rats subjected to stress (Fig. 4) and hypophysectomized animals injected intravenously with a large dose of ACTH (Fig. 5). In contrast, corticosterone secretion was relatively unstimulated for the first 15 minutes of collection, reached a maximum in the 15 to 30 minute period, and continued unabated over the remainder of the 1 hour collection period in both groups of rats (Figs. 4 and 5). The continuous and good blood flow from the cannulated renal vein is also shown in these figures. The rapid appearance of ascorbic acid in the adrenal venous effluent following stress and the only minimal release observed thereafter, combined with the grossly elevated systemic levels of ascorbic acid at later periods following the onset of stress, may explain the negative results of Vogt (6), who reported the absence of "adrenal" arterio-venous differences (carotid artery vs. adrenal vein) for ascorbic acid in the dog.

The relationship of ascorbic acid in the adrenal gland to the hormonal activity of this structure is obscure. Ascorbic acid has variously been implicated in the oxidative biosynthesis of the corticosteroids (cf. 18), the protection of adrenal enzymes active in corticosteroidogenesis from oxidative inactivation (cf. 4), and as a component part of the storage form of corticosteroids in the adrenal gland (19). The present finding that the vitamin was not destroyed during enhanced adrenal cortical activity would seem to cast doubt on the importance of the first two phenomena. However, simple transformation between ascorbic acid and dehydroascorbic acid could be involved and would not affect the chemical recovery of this vitamin. The observation of Vogt (6) that corticosteroids do not appear to be released by the cat adrenal in combination with ascorbic acid is consistent with the present finding of a dichotomy in time between the secretion of these two substances by the adrenal gland, at least in the rat. These results do not rule out the possibility that such a complex may exist preformed in the gland (19), may be split during adrenocortical activation, and its component parts released at different rates. It should also be pointed out that ACTH evokes the release of corticosteroids by the adrenal gland of the hypophysectomized dog within 2 minutes after injection (20) in contrast to the delayed secretion noted in the rat. Finally, it is conceivable that no essential relationship exists between ascorbic acid and corticosteroid metabolism in the adrenal gland (cf. 21) and that adrenocortical activation enhances the permeability of the adrenal cell (cf. 4), to both ascorbic acid and corticosteroids. In any event, although ascorbic acid depletion in the adrenal may be an indirect measure of corticosteroid secretion, it cannot necessarily be considered an indirect action of ACTH.

SUMMARY

The fate of ascorbic acid disappearing from the adrenal gland was studied in normal rats subjected to the stress of laparotomy and in hy-

pophysectomized rats injected intravenously with a large dose of ACTH (0.5 I.U. per 100 gm. body weight). In both groups of animals the amount of this vitamin lost by the cannulated adrenal gland could be quantitatively recovered in the venous effluent. The release of ascorbic acid was essentially complete within 15 minutes under these circumstances of acute (and, probably, maximal) adrenocortical activation. In contrast, corticosterone secretion was relatively non-stimulated for the first 15 minutes but reached a maximum in the 15 to 30 minute period. The possible relationship of adrenal ascorbic acid to corticosteroid metabolism and ACTH action is discussed.

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NOTES AND COMMENTS

THE INFLUENCE OF CORTISONE ON THE ACTION OF AN ADRENOCORTICOLYTIC DRUG[1]

Recent work has demonstrated that the drug DDD[2](2,2 bis-parachlorophenyl-1,1 dichloroethane) is adrenocorticolytic. Brown *et al.* (1) found that dogs fed DDD excreted almost no steroids of adrenal cortical nature and that the decrease in steroid excretion paralleled the degree of destruction of the adrenal cortex. In a later report (2) the same authors found that dogs fed DDD developed all the signs of adrenal cortical failure including decreased steroid excretion, increased insulin sensitivity, decreased eosinophil response to ACTH, muscular weakness, some impairment of salt metabolism, and decreased plasma steroid levels. The situation was not improved by the administration of ACTH during or following the period of DDD administration.

An obvious question then arose: Will the administration of an adrenal cortical steroid such as cortisone prevent the development of adrenal cortical failure, or alter the response to DDD? The following is a study of this question.

METHODS

The methods have been described in detail (2). A brief summary follows: Four healthy dogs with no evidence of adrenal cortical impairment as determined by excretion of steroids, eosinophil response to ACTH, insulin sensitivity, and ability to handle a salt load were used in the experiment. Two of the dogs were given DDD alone and two were given DDD plus 10 mg. of cortisone (Upjohn) subcutaneously per day. The DDD was administered in a gelatin capsule at the time of feeding at a constant level of 85 mg./kg./day. Tests of adrenal cortical function were made at regular intervals in the order previously described (2).

RESULTS AND DISCUSSION

In less than 3 weeks the dogs fed DDD alone refused to eat, became listless, developed muscular weakness and lapsed into coma. At this time the insulin sensitivity was about 10 times normal, the 17-hydroxy steroid excretion was almost zero as measured by the Porter-Silber reaction, and the administration of ACTH failed to elicit a drop in eosinophils. One dog died on the 17th day of DDD administration (Section of adrenal shown in Fig. 2), and on the 22nd day the second dog was comatose, cold, and near the point of death. At this time 100 ml. of 10% glucose was administered intravenously along with 10 mg. of cortisone. Within 12 hours the dog had recovered from the coma and was again on his feet. Cortisone was continued in a daily dose of 5 mg. by subcutaneous administration. Within three days the dog appeared perfectly normal, the lost weight was returning, and the appetite was normal.

During this same period of time the two dogs receiving the same dose of DDD with 10 mg. of cortisone per day (subcutaneously) remained externally normal. The dogs appeared lively, ate normally, and maintained weight. The excretion of steroids was high and the insulin sensitivity was low as would be expected from the large dose of steroid. The fasting blood sugar averaged about 135 mg. % as compared to the normal of about 80 mg. %. The dogs continued to appear externally normal during a period of six weeks during which the cortisone and DDD was continued in daily dosage. (In

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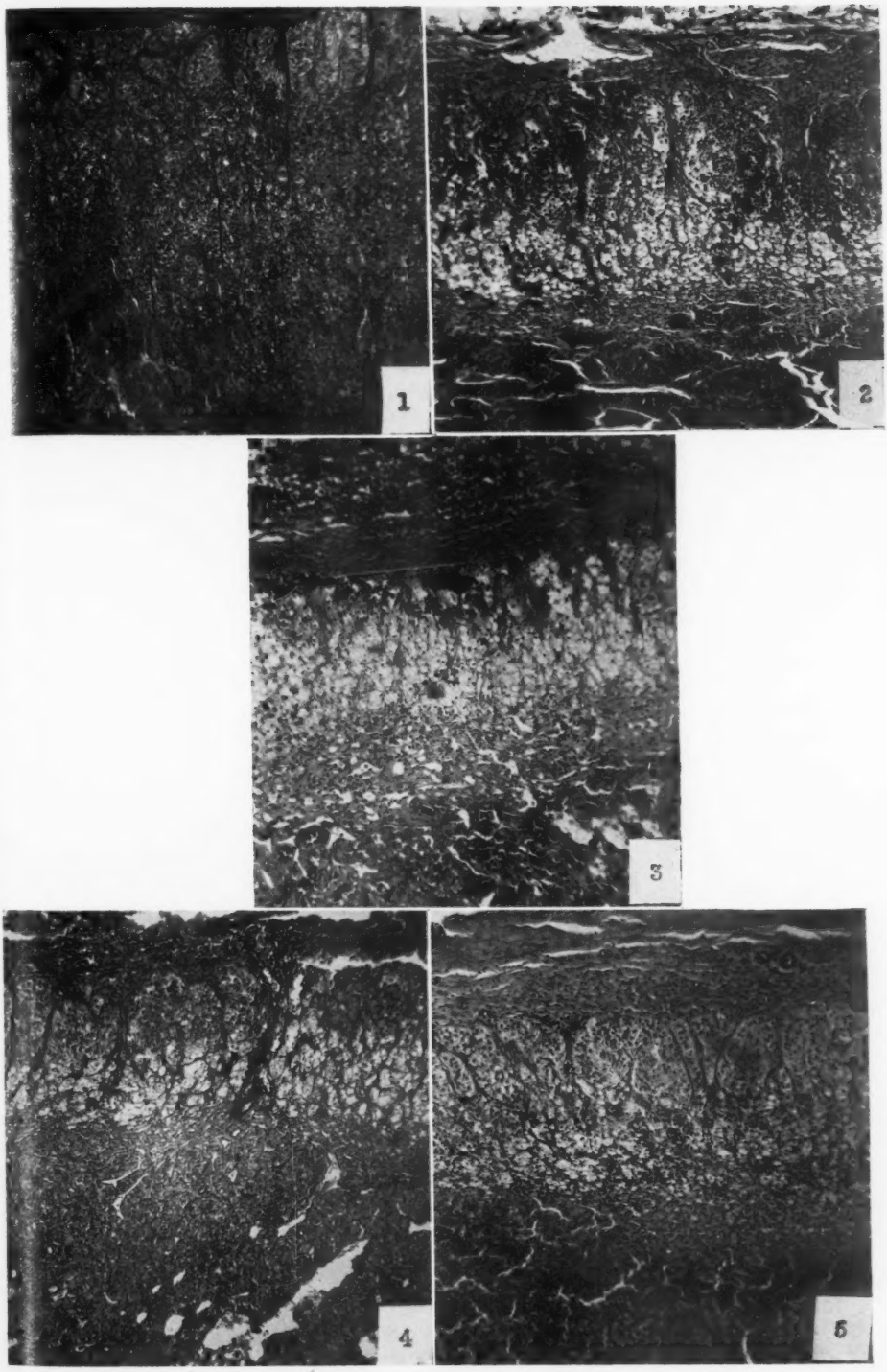
[1] Supported by a grant (CY-3150) from the USPHS, National Cancer Institute.

[2] DDD was supplied through the courtesy of Rohm and Haas, Philadelphia.



FIGURES 1–5. (*Explanation given on next page*)

previous experiments, 12 dogs fed DDD alone died in less than four weeks at this dose level.) After six weeks one of the dogs was sacrificed and the adrenal gland was taken for sections.[3] The cortex was necrotic with fatty infiltration. The gland weighed 95 mg./kg. body weight compared with a normal of 150 mg./kg. body weight. A typical area of the gland is shown in Figure 3. The second dog on DDD and cortisone received a final subcutaneous injection of 10 mg. of cortisone and was then taken off all treatment. This dog did not go into adrenal failure. The most probable explanation is the regeneration of the gland during the time before all of the implanted cortisone was absorbed. The dog which received DDD alone to the point of death and was restored by the administration of cortisone likewise did not develop adrenal failure when cortisone was stopped. Examination of the adrenal glands of these dogs shows intensive but not normal regeneration (Figs. 4, 5).

The well being of the dogs given DDD and maintained on cortisone demonstrates that the toxicity of the drug is due solely to its effect on the adrenal cortex. Microscopic examination of the liver and kidney of DDD-treated animals failed to show any evidence of tissue damage. The adrenal medulla is not involved. Previous work (2) has demonstrated that ACTH has no effect on the adrenal treated with DDD.

SUMMARY

Cortisone can replace the normal secretion of the dog adrenal cortex destroyed by DDD. In dogs fed DDD the adrenal cortex is destroyed but the physical signs of destruction are modified by simultaneous administration of cortisone. When treatment is halted, the gland regenerates and function returns, but the normal histological picture is not obtained.

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[3] The slides were examined by Dr. Abner Golden, Associate Professor of Pathology, Emery University.

The adrenal glands of dogs treated with DDD. All sections are stained with hematoxylin and eosin and are photographed at 80×. In each case the medulla is at the bottom of the photograph.

Fig. 1. The normal adrenal gland of the dog.

Fig. 2. Adrenal gland of dog treated with DDD for three weeks. Notice the extensive necrosis and loss of structure.

Fig. 3. Adrenal gland of dog treated with DDD for three weeks with the simultaneous administration of 10 mg. cortisone per day. This gland also shows extensive degeneration but the effect appears not as marked as in (2) above. There are a few intact glomerulosa cells.

Fig. 4. Adrenal gland of dog treated with DDD for three weeks, given cortisone to carry past adrenal cortical crisis and then allowed to remain without treatment for two weeks. Note the extensive regeneration in the glomerulosa in particular as well as in the other zones. This is not, however, a normal configuration.

Fig. 5. Adrenal gland of a dog treated with DDD and cortisone for 3 weeks after which all treatment was stopped. The gland was allowed to regenerate for 3 weeks. Note the abnormal regeneration very similar to that in (4).

ADDENDUM: Recent experiments similar to those described above have revealed that the adrenocorticolytic agent is a contaminant of the DDD. Highly recrystallized DDD did not cause adrenal damage after 8 weeks of feeding and was completely nontoxic while materials crystallized from the supernatant after DDD recrystallization were highly damaging to the adrenal. Chromatographic separations are now being made to obtain the active principle.

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PRODUCTION OF PORTER-SILBER REACTIVE MATERIAL BY HUMAN PLACENTA INCUBATED *IN VITRO*[1]

Extracts of human placentas, like glucocorticoids, promote liver glycogen deposition in adrenalectomized rats (1). Recently several corticosteroids including cortisol, cortisone and tetrahydrocortisone have been isolated from human placenta (2). This note describes an apparent synthesis of free 17-hydroxycorticosteroid by the human placenta, which may contribute to the elevated plasma concentrations which have been observed during pregnancy (3).

MATERIALS AND METHODS

Eleven term placentas were obtained at delivery; maternal and fetal umbilical cord blood samples were obtained at the same time and the sera were separated. The placentas were rinsed in cold water, and 20 grams were cut into small pieces, washed in saline and blotted. Two 10.0 gm. portions were minced into separate flasks, each containing 45 ml. Krebs-Ringer solution buffered with phosphate at pH 7.4, and 5 ml. of maternal serum. One flask was incubated in a water bath at 37° C. A fine stream of air was bubbled through it, and it was shaken intermittently by hand. The supernatant fluid in the other flask was decanted immediately, and the tissue residue was homogenized in 50 ml. Krebs-Ringer solution and centrifuged. The decanted medium and the supernatant fluid from the centrifuged homogenized placental tissue from each incubated and nonincubated flask was assayed for 17-hydroxycorticosteroid by the Nelson and Samuels method (4), using crystalline cortisol as a standard.[2] The experimental procedure is summarized in Table 1. Ten gm. of each placenta were also homogenized immediately in Krebs-Ringer solution, and analyzed to give control values for the total placental content. This procedure analyses for the concentration of the water soluble free 17-hydroxycorticosteroid in the tissue and in the incubation medium before and after incubation.

RESULTS

Eleven incubations were performed. The placental residue before incubation contained a mean total concentration of 11.0 μg. of 17-hydroxycorticosteroid per 10.0 gm. wet tissue. After incubation the mean total concentration was 7.3 μg. This difference is not statistically significant. The medium before incubation contained a total of 4.5 μg. 17-hydroxycorticosteroid (corrected for the maternal serum added). After incubation the medium contained a total of 10.7 μg., a net increase of 6.5 μg., which is statistically significant ($P < 0.02$).

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[1] This work was supported by the Josiah Macy, Jr. Foundation.

[2] Supplied by Merck and Company.

TABLE 1

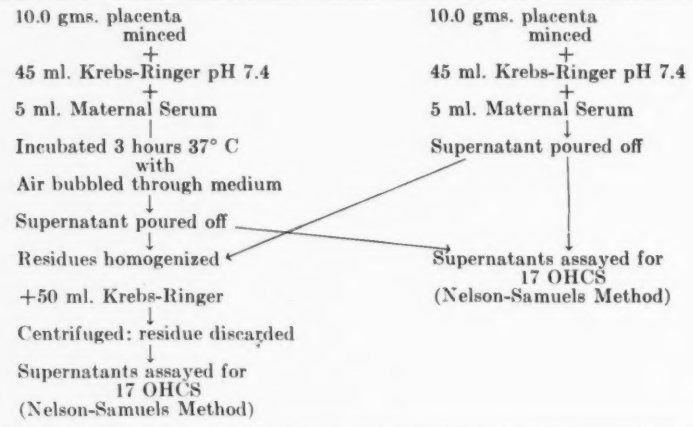

TABLE 2. 17-HYDROXYCORTICOSTEROID IN 11 PLACENTAL INCUBATIONS

	Before incubation	After incubation
Medium	4.5 ± 1.9	10.7 ± 3.7
Placenta	11.0 ± 4.1	7.3 ± 3.3

Mean values expressed as μg. per total volume of medium or water soluble homogenate ± standard error of mean.

MATERNAL AND FETAL SERUM 17-HYDROXYCORTICOSTEROID CONCENTRATIONS

Maternal (11)	42.4 ± 14.9
Fetal Umbilical Cord (8)	17.7 ± 5.1

Mean values expressed as μg. per 100 ml. serum ± standard error of mean.

The mean concentration in the maternal serum of these eleven patients was 42.4 μg per 100 ml. serum and eight of the eleven umbilical cords had a mean concentration of 17.7 μg. per 100 ml. serum which are consistent with results previously obtained in this laboratory.

The total mean concentration of 10.0 grams of each placenta taken as control was 15.5 μg. (duplicate determinations).

DISCUSSION

The results show an increase of 17-hydroxycorticosteroid in the medium after placental incubation *in vitro*, without any significant loss from the placental tissue itself.

The observed increase may be the result of metabolism of other steroids by the term placenta to compounds which give a positive Porter-Silber reaction, it may be hydrolysis of conjugated steroids, or it may be the simple liberation of tightly protein-bound 17-hydroxycorticosteroid, which was not freed by chloroform extraction alone.

The observed concentration of the nonincubated medium may be extracellular 17-hydroxycorticosteroid, washed out after mincing the placenta. This is confirmed by the mean concentration of the nonincubated placenta and its medium, being equal to the mean concentration of the control 10.0 gm. of placental tissue.

SUMMARY AND CONCLUSIONS

1. Eleven term placentas were incubated in Krebs-Ringer solution and maternal serum from the same patient. Estimations of free 17-hydroxycorticosteroid by the Nelson and Samuels method were made of the medium and the placental tissue before and after incubation.

2. An increase of 17-hydroxycorticosteroid was demonstrated in the medium after incubation, without a significant loss from the placental homogenates.

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THE EFFECT OF THYROTROPIN ON THYROGLOBULIN HYDROLYSIS[1]

In a previous communication (1) a method was described for the hydrolysis of I^{131}-labelled rat thyroglobulin by enzyme(s) present in saline extracts of sliced thyroid glands. The effect of adding thyrotropin (TSH) to this *in vitro* reaction is now reported.

Three preparations of TSH were used.[2] Saline extracts of labelled rat thyroid glands were prepared as previously described (1). Various dilutions of each TSH preparation were made in acetate buffer, pH 4.90, and mixed with equal volumes of the thyroid extract to form reaction solutions. The pH of each reaction solution was verified before incubation to be 4.90 ± 0.02. The solutions were incubated at 37° C and samples were removed at intervals. The extent of hydrolysis was measured by dialysis against normal saline, as previously described (1).

The results, as indicated in the table, show that in the control samples with no TSH, the dialyzable radioactivity was approximately twice as great at 24 hours as at 6 hours. TSH preparation #1, in increasing concentrations, caused an increase in hydrolysis after 6 hours' incubation, but this enhancing effect was no longer discernible at 24 hours. TSH preparation #2 exerted a consistent inhibitory effect on the reaction. With concentrations of 5,000 and 10,000 μg. per ml. there was negligible hydrolysis at 6 hours. At 24 hours, hydrolysis in the presence of this preparation showed an increase of 2–5 fold over the six-hour values, but was still depressed below the 24-hour controls without TSH. TSH preparation #3 appeared to exert no effect whatever on the reaction.

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[2] Preparation #1: Thytropar (Thyrotropin Armour), approx. 2.5 U.S.P. Units/mg. Preparation #2: Parke, Davis #099802, approx. 5–10 U.S.P. Units/mg. Preparation #3: Armour #317–51, approx. 5–10 U.S.P. Units/mg.

TABLE 1. PER CENT OF DIALYZABLE I^{131} IN REACTION SOLUTIONS AFTER 6 AND 24 HOURS' INCUBATION WITH VARYING CONCENTRATIONS OF TSH PREPARATIONS

TSH μg./ml.	6 hrs. incub.			24 hrs. incub.		
	Prep. #1	#2	#3	Prep. #1	#2	#3
0	21.9	27.9	30.3	51.3	56.7	61.6
100	26.2					
500		19.9	20.3		47.1	63.2
1,000	36.5	10.6	35.0	47.0	43.4	65.5
5,000		5.2	32.4		30.2	68.5
10,000		5.3	31.2		29.6	60.4

It is concluded that TSH per se exerts no influence on the *in vitro* proteolysis of thyroglobulin under the conditions described. The enhancing effect of TSH preparation #1 was evident only in the earlier (6-hour) sampling period, and this is in the sensitive portion of the time curve for this mode of thyroglobulin hydrolysis (1). However, since the other TSH preparations did not exert a similar effect, the result with preparation #1 cannot be attributed to its content of TSH. It may indicate the presence in TSH preparation #1 of a non-specific cathepsin, since it has been shown that TSH preparations may contain measurable catheptic activity using hemoglobin as the substrate (2). TSH preparation #2 might inhibit hydrolysis of thyroglobulin by competing with it for the proteolytic enzyme. There is, however, no evidence for such an effect with the other two preparations.

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IN VIVO METABOLISM OF ESTRADIOL-17β-16-C^{14} IN THE HUMAN BEING: ISOLATION OF 2-METHOXYESTRONE-C^{14} [1]

The recent announcement by Kraychy and Gallagher (1) of the isolation of 2-methoxyestrone from human urine prompts us to report our own observations which confirm and to some extent supplement the findings of these authors.

Estradiol-17β-16-C^{14} [2] (100 mg., specific activity 1.16×10^5 cpm./mg.) was administered intravenously in propylene glycol-human serum albumin[3] to a post-menopausal woman with cancer of the breast. Urine was collected on the day of the infusion and for six days thereafter and stored in the frozen state until it was processed. A methylene

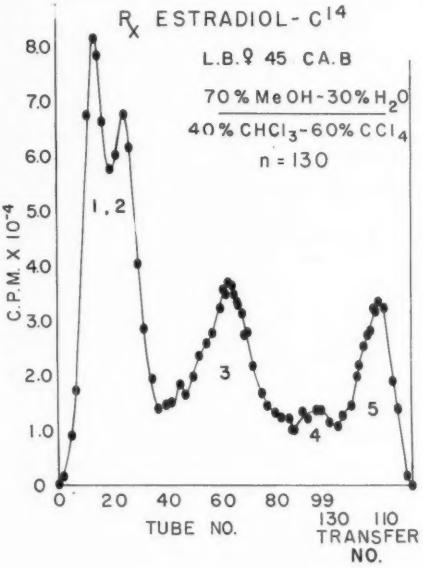

FIG. 1. Counter-current distribution (130 transfers) of urinary extract after administration of estradiol-17β-16-C^{14}. Peak 1 contains 2-methoxyestrone, Peak 2 estrone, Peak 3 estradiol, Peak 4 unidentified metabolites, and Peak 5 estriol and other highly polar metabolites.

chloride extract of the urine was prepared after hydrolysis of the steroid conjugates with β-glucuronidase.[4] The initial purification of the crude extract consisted of two short countercurrent distributions, the first between hexane and 90% aqueous methanol and the second, between ethyl acetate and water. Following these steps the extract was subjected to a 130-transfer counter-current distribution in 70% methanol-30% H_2O as upper phase and 40% $CHCl_3$ −60% CCl_4 as lower phase (Fig. 1). In addition to the

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[2] Charles E. Frosst & Company, Montreal, Canada.

[3] We wish to express our thanks to Dr. William H. Baker for carrying out this phase of the experiment.

[4] Ketodase, Warner-Chilcott Laboratories, New York.

TABLE 1. PARTITION COEFFICIENTS OF ESTRONE AND 2-METHOXYESTRONE

System[1]		Partition coefficient[2]	
Upper phase	Lower phase	Estrone	2-Methoxyestrone
70% CH_3OH-30% H_2O	40% $CHCl_3$-60% CCl_4	0.24	0.12
70% CH_3OH-30% H_2O	CCl_4	1.05	0.24
$C_6H_5CH_3$	1N NaOH	0.14 (3)	1.20

[1] The solvent systems were prepared by equilibrating equal volumes of the two phases.
[2] The partition coefficient (K) is defined as concentration of solute in upper phase/concentration of solute in lower phase.

three major components, estrone, estradiol and estriol, and some as yet unidentified hydrophilic substances, a zone of radioactive material less polar than estrone was observed. This substance was separated cleanly from estrone and from a minor component by a second counter-current distribution of 130 transfers between 70% methanol-30% water as upper phase and CCl_4 as lower phase. The new radioactive material was then separated from hydrophilic phenolic and acidic material by a counter-current distribution with complete stripping (2) between toluene and 1N NaOH. Five portions each of upper and lower phase were used and a phase volume ratio of 5:1 was employed. Organic material was recovered from the alkali phase by adjustment of the pH to 8.5–9.0 and ether extraction (3). The radioactivity remained in the toluene phase. Finally, the new substance was purified by a twenty-four transfer counter-current distribution between toluene and 1N NaOH using a phase-volume ratio of 0.5 which separated it from a small amount of an even more hydrophobic substance. The partition coefficients are summarized in Table 1. The major component upon paper chromatography in a modification of the Bush (4) toluene-ligroin (67–33)-methanol-water (70–30) system and radioautography gave a single radioactive zone (R_f 0.74) which corresponded in position to the zone which gave a positive Zimmermann reaction and a positive reaction with the Folin-phenol reagent as modified by Mitchell (5). Comparison of the infra-red spectrum of material from the peak tube of the final counter-current distribution with that of synthetic 2-methoxyestrone showed complete identity.[5]

In this experiment, 2-methoxyestrone was by no means a minor metabolite; over the seven days during which collections were made, this compound accounts for roughly 8% of the administered radioactivity. This fact in addition to the unexpected chemical nature of the metabolite emphasizes its importance in the overall picture of estrogen metabolism.

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[5] The infrared spectrum was measured by Mrs. D. Wheeler and the comparison was performed through the courtesy of Dr. T. F. Gallagher.

THE ENDOCRINE SOCIETY

1957 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Medal of The Endocrine Society

In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. Dr. Carl R. Moore was the recipient in 1955, Dr. Frederick L. Hisaw in 1956, and Dr. Joseph C. Aub in 1957.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl Heller; 1949—Dr. George Sayers; 1950—Dr. Oscar M. Hechter; 1951—Dr. Albert Segaloff; 1952—Dr. Seymour Lieberman; 1953—Dr. Sidney Roberts and Dr. Clara M. Szego (Mrs. Roberts); 1954—Dr. I. M. Rosenberg; 1955—Dr. Jack Gross; 1956—Dr. Alfred M. Bongiovanni; 1957—Dr. Nicholas S. Halmi. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-four months of the date of the award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and The Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually for each individual and shall be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Medal of the Endocrine Society; the Ciba Award; the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *November 1* each year.

Proposals for appointment as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *November 1, 1957.*

The Awards Committee will meet in November and notice of awards to successful nominees and applicants will be made not later than December 1.

Citation for the 1957 Award

The Medal of the Endocrine Society

Dr. Joseph Charles Aub, past president of the Endocrine Society, has been a continuous contributor to endocrinology throughout his long and active life. Born in 1890 in Cincinnati, Ohio, he graduated with an A.B. degree from Harvard College in 1911 and an M.D. degree from Harvard Medical School in 1914. Following a medical internship at the MGH, with Dr. Eugene DuBois he worked out the surface area formula for the calculation of metabolic rate, with Dr. Dennis the disturbed glucose metabolism of the patient with hyperthyroidism. With the research group in France during World War I he described the lowered metabolic rate of the state of shock. After the war as an Assistant Professor of Physiology he made his classic studies in lead poisoning. He and his co-workers described the passage of lead with calcium into and out of the bones. These studies laid the foundation not only for the modern therapy of lead poisoning but also for the clinical studies of calcium and phosphorus metabolism. With Dr. DuBois and their colleagues he described the first case of hyperparathyroidism in this country at a time when the discovery of Mandl was still unknown. He was also the first to recognize the effect of thyroid and pituitary hormone on calcium metabolism. His earlier studies in calcium led on to that of radium and other heavy metals.

In 1928 he was appointed Director of the Huntington Laboratories of Cancer Research at Harvard University and was one of the first to approach the problem of cancer from the point of view of endocrinology. Hormone studies were made of the growth and development of children and of patients suffering from various cancers. With Dr. Ira Nathanson he was in the forefront of treating breast cancer with hormones.

In 1942 when the Huntington Laboratories moved to the MGH he was promoted to Professor of Research Medicine. The endocrine studies of cancer continued under his direction. His publications now numbering 176 cover a wide range of subjects involving growth and metabolism. Retired from his chair of medicine in 1956 he has returned to the laboratory to study the cell membrane.

In order to judge the fullness of his accomplishment one has to look at the long list of investigators who at one time or another have studied under him and have been inspired by him. Many are members of the Endocrine Society and many are professors of medicine in medical schools throughout the U. S. and Canada. His greatest interest has been in these young colleagues and to them he has brought the great warmth of his personality.

Citation for the 1957 Ciba Award

The Ciba Award for 1957 goes to Dr. Nicholas S. Halmi, who is a worthy addition to the group of young endocrinologists so honored by the Endocrine Society.

Dr. Halmi's capacity for acute perception, objective reasoning, and fluent expression are native endowments derived from an equally gifted father. The manner in which he has employed those talents to carve for himself a brilliant career in science must be credited to himself alone.

Dr. Halmi was born in Budapest, June 6, 1922, where he received his primary and secondary education. He attended medical school at the Universities of Kolozsvár (Chuj), Transylvania, and Budapest, where he graduated "sub laurea Almae Matris" in 1947, despite the fact that during the German occupation of Hungary he was taken into custody by the Gestapo and after his release was obliged to go underground. During his intern training in Budapest, he also worked for two years as a research assistant in pathology at St. Johns Hospital and in 1947 became assistant to Professor J. Szentagothai in the Department of Anatomy, University of Pecs, where his interest in the study of hypophysio-hypothalamic relationships was born. Dr. Halmi and his father, who had been prominent in the financial and cultural affairs of pre-Communist Hungary, were under constant surveillance by the Communists during this period. In January, 1949, the pressure became very acute and after it became evident that they were about to be deported to Siberia, the Halmis escaped in a commandeered plane to Munich and later that year Dr. Halmi reached the United States. In 1953 he became a naturalized citizen.

From 1949–1950 Dr. Halmi was an instructor in anatomy at the University of Chicago. Since 1950 he has been a member of the Department of Anatomy, State University of Iowa, where he now holds the rank of Associate Professor and where he is recognized as a gifted and stimulating teacher, as well as a brilliant investigator.

Dr. Halmi's studies have been focussed primarily in the area of his early interest in hypophysio-hypothalamic relationships, but his inquiring mind has ever turned to new methods of approaching this complex subject, as well as other problems of endocrine physiology. Thus his interests have included various studies on the cytophysiology of the hypophysis and hypothalamus, on the effect of hypothalamic lesions in various aspects of hypophysial function, on the relation of hormonal and dietary factors to carbohydrate metabolism, and on the relation between intrinsic and hypophysial factors in the regulation of the iodide concentrating mechanisms of the thyroid. Although his more recent work has been in other areas, Dr. Halmi continues to be regarded as an authority on cytophysiology of the hypophysis. His contributions to this subject included the recognition of differences in morphology and function of the beta and delta cells of the

rat pituitary, as well as clearly defined demonstrations of the response of the pituitary to a variety of experimental conditions. His studies on modification of hypophysial function by lesions in the hypothalamus ranged through the entire endocrine system and were particularly valuable in regard to effects on carbohydrate metabolism, and thyroid and gonadal function. Some of these findings led to important studies on the relation of dietary and hormonal factors to carbohydrate metabolism. By no means the least significant of his investigations have been those concerning the iodide concentrating mechanisms of the thyroid. His work on iodide trapping as influenced by serum iodine levels and thyrotrophic hormone represents an important advance in our understanding of thyroid physiology and has become the basis of a widely used technique in studies of thyroid function. During the last six years, he has published approximately thirty papers, each of which has represented a carefully executed study reported in a skillful and meticulous fashion. His command of our language is such that his American-born colleagues have eagerly accepted his suggestions for changes in their own manuscripts.

Dr. Halmi's contributions to the advance of experimental endocrinology have been significant and valuable both for their content and for the stimulus they have provided to further investigations. We may be certain that his capacity for original thinking and for skillful execution of his ideas will combine to provide us with many more important studies.